REMARKS

A. Disposition of Claims

Claims 1-24 are pending in this application. Claims 1, 3-7, 9-15 and 21-24 were withdrawn from consideration. Claims 16 and 20 are amended herewith to conform to the preferred embodiment, and thus for reasons unrelated to patentability. Support for the amendment is located throughout the specification, for example, at original Claims 16 and 20. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

B. Compliance with 35 USC 112/2

The issue is whether Claim 16 is in compliance with 35 USC 112/2 as being definite in particularly pointing out and distinctly claiming the subject matter that Applicant regards as the invention. In Claim 16, the claim recites "substantially all of the nonstructural region". It is said to be unclear what applicant intends by "substantially all". Claim 16 has been amended to recite "the whole nonstructural region" to conform to the preferred embodiment. The conclusion is that any perceived ambiguity has been resolved and that Claim 16 is in compliance with 35 USC 112/2 as being definite.

C. Compliance with 35 USC 112/1 enablement

The Patent Office rejected Claims 16 and 20 under 35 USC 112/1 as failing to meet the enablement requirement. Under MPEP 2164, the test for enablement is whether one skilled in the art could make or use the subject matter defined by the claims without undue experimentation. Claims 16 and 20 were rejected because the specification, while agreed to be enabling for the whole 5' UTR; the whole NS region; the whole 3' UTR; and all of the structural proteins to encapsulate the subgenomic replicon, was said not to reasonably provide enablement for a mere part of the 5' UTR, just a part of the NS region, a mere part of the 3' UTR, or less than that full complement of structural proteins for encapsulating said replicon. Claim 16 has been amended to recite "the whole 5'UTR", "the whole nonstructural region" and "the whole 3'UTR", and Claim 20 has been amended to recite "each of the structural proteins", all to conform to the preferred embodiment. The conclusion is that the specification is enabling with respect to the

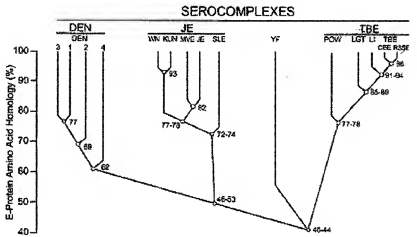
claimed subject matter and that Claims 16 and 20 are in compliance with 35 USC 112/1 as meeting the enablement requirement.

D. Compliance with 35 USC 103(a)

The issue is whether the claims are in compliance with 35 USC §103(a) or unpatentable over WO 99/28487 to Westaway et al., Ref. 1, in view of Schlesinger et al., J. Gen. Virol. 68: 853 (1987), Ref. 27, Bartenschlager, [citation unknown], and Fields Virology 3rd ed., Philadelphia, Pa., Lippincott-Raven Publishers, pp. 931-959 (1996), Ref. 26, and Claim 16 further in view of Khromykh & Westaway, J. Virol. 71: 1497 (1997), Ref. 14.

WO9928487 describes construction of subgenomic replicons of Kunjin virus and their packaging into virus-like particles by a packaging cell line. Schlesinger et al. describes protection of mice against DEN 2 virus encephalitis by immunization with DEN 2 non-structural glycoprotein NS1. Bartenschlager is said to describe experiments with HCV. Varnavski & Khromykh, Virology 255: 366 (1999) and Khromykh & Westaway describe additional experiments with Kunjin. Behrens et al., J. Virol. 72: 2364 (1998) describes experiments with BVDV. Fields is said to teach that flaviviruses (of which dengue is a species) have structurally similar genomes.

Nevertheless, the construction of subgenomic replicons of Dengue virus and their packaging into virus-like particles could not have been predicted from WO9928487 either singly or in combination with the other cited art. This is because, first, Kunjin belongs to the Japanese encephalitis virus group of flaviviruses whereas Dengue virus belongs to the different Dengue virus group.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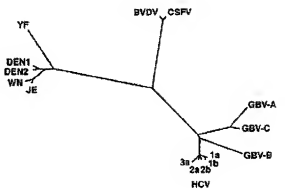
50
40
93
92
77-79
72-74
77
88
82
45-53
77-78
96
91-94
85-89
48-44

As shown in the art of Fields Virology 3rd ed., Philadelphia, Pa., Lippincott-Raven Publishers, pp. 961-1034 (1996) at Fig. 2, above, full citation attached, the Japanese encephalitis virus group and Dengue viruses share only 46-53% amino acid sequence homologies as exemplified by E protein. Consequently, while flaviviruses share some common features with regard to genome organization, they share little sequence homology. Thus the construction of subgenomic replicons of Dengue virus and their packaging into virus-like particles could not have been predicted from the work with Kunjin virus.

Additionally, the construction of subgenomic replicons of Dengue virus and their packaging into virus-like particles could not have been predicted because, second, HCV and BVDV are not flaviviruses. Rather, they are members of the family Flaviviridae. HCV is not a flavivirus, instead it is a hepatitis C virus (Fields, Ref. 26, Table 1). BVDV is not a flavivirus, rather it is a pestivirus (Fields, Ref. 26, Table 1).


BVDV
CSFV
YF
DEN1
DEN2
WN
JE
GBV-A
GBV-C
GBV-B
1a
1b
3a
2a
2b
HCV

As shown in the art of Fields Virology 4th ed., Philadelphia, Pa., Lippincott Williams & Wilkins Publishers, pp. 991-1041 (2001) at Fig. 1, above, full citation attached, the three genera, the flaviviruses (of which Dengue virus is a member), the pestiviruses (of which BVDV is a member), and the hepaciviruses (of which HCV is a member), are phylogenetically diverse. Only Kunjin is a flavivirus, but as explained above, Kunjin belongs to the Japanese encephalitis virus group of flaviviruses whereas Dengue belongs to the different Dengue virus group. Thus the additional references do not assist in the construction of subgenomic replicons of Dengue virus and their packaging into virus-like particles.

At the time of the filing date, the development of flavivirus replicons had been investigated only for Kunjin. However, members of the flavivirus genus are quite diverse, as described above, and as evidenced by Khromykh et al. 1997, Ref. 14, on page 1504, col. 1, first paragraph, in which the authors report that a direct comparison between Kunjin and Dengue is difficult because of genetic heterogeneity. Therefore, the development of replicons from other flavivirus members, specifically dengue virus that demonstrates significant phylogenetic divergence, could not have been discerned in the absence of known properties common to the genus flavivirus from those specific to the particular member Kunjin. For these reasons, the conclusion is the claims are patentable over any prior art and in compliance with 35 U.S.C. §103(a).

E. Compliance with Rules Against Double Patenting

The Patent Office provisionally rejected certain of the pending claims under the judicially created doctrine of obviousness-type double patenting as being unpatentable over selected claims of U.S. Pat. Appl. No. 11/192,923, filed July 29, 2005, or U.S. Pat. Appl. No. 11/194,342, filed still later based on its Serial No. The rule according to MPEP 804 I B 1 is that if a "provisional" obviousness-type double patenting rejection is the only rejection remaining in the earlier filed of the two pending applications, while the later-filed application is rejectable on other grounds, the examiner should withdraw that rejection and permit the earlier-filed application to issue as a patent without a terminal disclaimer. Where there are three applications containing claims that conflict such that an obviousness-type double patenting rejection is made in each application based upon the other two, it is not sufficient to file a terminal disclaimer in only one of the

applications addressing the other two applications. Rather, an appropriate terminal disclaimer must be filed in at least two of the applications to link all three together. Here, a "provisional" obviousness-type double patenting rejection is the only rejection remaining in the earliest filed of all three pending applications, because this application was filed September 5, 2003, while U.S. Pat. Appl. No. 11/192,923 was filed July 29, 2005 and U.S. Pat. Appl. No. 11/194,342 was filed even later based on its Serial No. Consequently, an appropriate terminal disclaimer can be filed in the two later-filed applications to link all three together if an obviousness-type double patenting rejection remains in the two later-filed applications. Thus, the examiner is respectfully requested to withdraw the rejection in this application and permit the earliest-filed application to issue as a patent without a terminal disclaimer.

CONCLUSION

Applicant respectfully requests that a timely Notice of Allowance be issued in this case. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 1/19/07

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Nancy W. Vensko
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VIROLOGY

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CHAPTER 31

Flaviviruses

Thomas P. Monath and Franz X. Heinz

The family *Flaviviridae* comprises 69 viruses, 67 of which are arthropod-borne viruses or close relatives of these arboviruses (Table 1). The family also includes simian hemorrhagic fever virus and hepatitis C virus (Chapters 30–32). Of the 67 arboviruses, 34 (50%) are mosquito-borne, 19 (28%) are tick-borne, 12 (18%) are zoonotic agents transmitted between rodents or bats without known arthropod vectors, and 2 have unidentified transmission cycles (Fig. 1). Thirty-eight viruses (55%) have been associated with human disease, including the most important arthropod-borne viral afflictions of humankind—dengue fever, yellow fever, and Japanese encephalitis (JE). Eight flaviviruses are pathogenic for domestic or wild animals of economic importance (Table 1).

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HISTORY

Yellow fever virus is the type virus of the family (hence the name, derived from *flavus*, Latin for "yellow"). It was the first filterable agent shown to cause a human disease and the first virus proved to be transmissible by an arthro-

TABLE 1. *The Flaviviridae (arboviruses): vector-host relationships and disease associations*

Virus	Vector[a]	Principal vertebrate host	Geographic distribution	Human disease	Animal disease
Absettarov[b]	Tick	Rodent	Eur.	+	
Alfuy	Mosquito	Bird	Austr.		
Apoi		Rodent	Asia	+[c]	
Aroa	?Mosquito		SA		
Bagaza	Mosquito		Afr.		
Banzi	Mosquito		Afr.	+	
Bouboui	Mosquito	?Monkey	Afr.		
Bussuquara	Mosquito	Rodent	SA	+	
Cacipacore		Bird	SA	+	
Carey Island		Bat	Asia		
Dakar bat		Bat	Afr.	+	
Dengue 1	Mosquito	Human, monkey	WW	+	
Dengue 2	Mosquito	Human, monkey	WW	+	
Dengue 3	Mosquito	Human	WW	+	
Dengue 4	Mosquito	Human	WW	+	
Edge Hill	Mosquito	?Marsupial	Austr.	+	
Entebbe bat		Bat	Afr.		
Gadgets Gully	Tick	?Bird	Austr		
Hanzalova[b]	Tick	Rodent	Eur.	+	
Hypr[b]	Tick	Rodent	Eur.	+	
Ilheus	Mosquito	Bird	SA	+	
Israel turkey meningoencephalitis	Mosquito	Bird	ME, Afr.		Turkey
Japanese encephalitis	Mosquito	Bird, pig	Asia	+	Pig, horse
Jugra	Mosquito	Bat	Asia		
Jutlapa		Rodent	CA		
Kadam	Tick	?Rodent	Afr., ME		
Karshi	Tick	?Rodent	Asia		
Kedougou	Mosquito		Afr.	+	
Kokobera	Mosquito	?Bird	Austr.	+	
Koutango	Tick, (?Mosquito)	Rodent	Afr.	+	
Kumlinge[b]	Tick	Rodent	Eur.	+	
Kunjin	Mosquito	Bird	Austr.	+	Horse
Kyasanur Forest disease	Tick	Rodent	Asia	+	
Langat	Tick	?Rodent	Asia	+[d]	
Louping ill	Tick	Bird, rodent	Eur.	+	Sheep, pig, horse, goat, deer, dog, grouse
Meaban	Tick	Bird	Eur.		
Modoc		Rodent	NA	+	
Montana myotis leukoencephalitis		Bat	NA		
Murray valley encephalitis	Mosquito	Bird	Austr.	+	?Horse
Naranjal	Mosquito	?Rodent	SA		
Negishi	Tick		Asia	+	
Ntaya	Mosquito		Afr.		
Omsk hemorrhagic fever	Tick	Rodent	Asia	+	Muskrat
Phnom-Penh bat		Bat	Asia		
Powassan	Tick (Mosquito)	Rodent	NA, Asia	+	
Rio Bravo		Bat	NA	+	
Rocio	Mosquito	Bird	SA	+	
Royal Farm	Tick	Bird	Asia		
Russian spring-summer encephalitis	Tick	Rodent, bird	Asia, Eur.	+	
Saboya	?Phlebotomine	Rodent	Afr.		
St. Louis encephalitis	Mosquito (Tick)	Bird	NA, CA, SA	+	
Sal Vieja		Rodent	NA		
San Perlita		Rodent	NA		
Saumarez Reef	Tick	Bird	Astr.		
Sepik	Mosquito		Afr.	+	
Sokuluk		Bat	Asia		
Spondweni	Mosquito		Afr.	+	

TABLE 1. *Continued*

Virus	Vector[a]	Principal vertebrate host	Geographic distribution	Human disease	Animal disease
Stratford	Mosquito		Austr.		
Tembusu	Mosquito	?Bird	Asia, Austr.		
Tyuleniy	Tick	Bird	Asia, NA		
Uganda S	Mosquito	Bird	Afr.		
Usutu	Mosquito	Bird	Afr.	+	
Wesselsbron	Mosquito (Tick)	?Rodent, sheep	Afr., Asia	+	Sheep
West Nile	Mosquito (Tick)	Bird	Afr., Eur., Asia	+	Horse
Yaounde	Mosquito	Rodent, bird	Afr.		
Yellow fever	Mosquito (Tick)	Monkey	Afr., SA	+	
Zika	Mosquito	Monkey	Afr., Asia	+	

[a]Parentheses indicate isolation from alternate vector, but uncertain role in natural transmission cycle.
[b]Viruses closely related or identical to Russian spring-summer encephalitis.
[c]Laboratory infection only.
[d]Disease following experimental infection for cancer therapy.
Afr., Africa; Austr., Australia-New Guinea; CA, Central America; Eur., Europe; ME, Middle East; NA, North America; SA, South America; WW, world-wide.
Modified from Karabatsos (263).

pod vector. These discoveries occurred on the threshold of the 20th century, some 350 years after the first clinical description of the disease. Yellow fever virus was the first flavivirus to be isolated (in 1927) and cultivated *in vitro* (in 1932). During the first decade of this century, dengue virus was also shown to be a filterable virus transmitted by arthropods, but it was not isolated until 1943.

A number of diseases characterized by meningoencephalitis were recognized as nosologic entities during the 19th and 20th centuries and later proved to be caused by flaviviruses. Among these are louping ill (a disease of sheep, recognized in Scotland since 1807), JE (Japan, 1873), and Australian X disease [now known as Murray Valley encephalitis (MVE), Australia, 1917]. Between 1931 and 1937 the viruses responsible for louping ill, St. Louis encephalitis (SLE), JE, and tick-borne encephalitis (TBE) were isolated. Common features of these viruses included neurotropism and arthropod transmission, but initially they were believed to be unrelated agents. In the late 1930s and early 1940s, relationships were demonstrated by neutralization and complement fixation tests between JE, SLE, and West Nile (WN) viruses. With the advent of the hemagglutination-inhibition test, which defined the broadest spectrum of antigenic relatedness, Casals and Brown (73) were able to separate the flaviviruses (group B arboviruses) from the alphaviruses (group A viruses) and to define the cross-reactions among a set of ten flaviviruses. As the flavivirus family grew with continued isolations of new agents from wild vertebrates and arthropods, so did the complexity of their serologic classification (72). Thirteen new flaviviruses were isolated for the first time during the 1970s, and one new virus has been recovered since 1980.

Notwithstanding the lack of a serologic relationship, the group A and B arboviruses were originally linked on the basis of their mode of transmission and physicochemical characteristics into a single family, the family *Togaviridae*, encompassing small RNA viruses with lipid envelopes and cubic nucleocapsid symmetry. As knowledge of the morphogenesis, biochemistry, and replication strategy of the flaviviruses expanded, it was clear by 1984 that their differences when compared to other togaviruses were sufficiently great to place them in a scparate family (587). Great strides have been made in the molecular characterization of flaviviruses in the last decade, including the elucidation of the genome organization and function, replication strategy, and crystallographic structure (see Chapter 30 and below).

INFECTIOUS AGENTS

Physical and Chemical Properties

Flavivirus morphology, morphogenesis, protein composition, and genome structure are described in detail in Chapter 30 [see also (462)]. Here we focus on the characteristics of viruses which relate either to interactions with the environment or to laboratory manipulations in antigen and vaccine preparation. In the next section, the chemical and physical properties of gene products interacting with antibody and immunologically specified cells are described.

Flavivirus virions consist of a spherical ribonucleoprotein core surrounded by a lipoprotein envelope with small surface projections. The projections seen in electron micrographs are clarified by X-ray crystallography and represent molecules of envelope glycoprotein, which form rodlike structures anchored to the viral membrane at their basal ends. Envelope lipids constitute approximately 17%

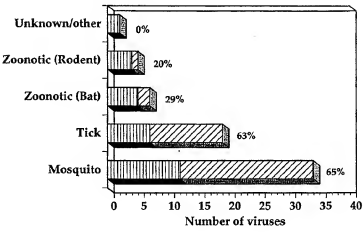


FIG. 1. Number of recognized flaviviruses, by mode of transmission. The number of viruses (*cross-hatched column*) and proportion (%) causing human disease in each transmission category are shown. Thre majority of viruses are mosquito- or tick-borne, and over 60% of these agents cause human illness.

of the virion dry weight (554) and are derived from the host-cell lipids. Lipases and lipid solvents disrupt flavivirus particles. Inactivation by chloroform and sodium deoxycholate provides a useful preliminary step in identifying flaviviruses (and other enveloped arboviruses). Acetone, which is often used to extract flavivirus antigens from infected mouse brain tissue, also destroys infectivity, whereas addition of sucrose partially preserves it. Treatment with beta-propiolactone is an effective inactivating procedure that retains flavivirus antigenic reactivity to a greater extent than does formalin or phenol treatment.

Mature flavivirus virions contain three structural proteins: a nucleocapsid or core protein (C; 12 kd), a nonglycosylated membrane protein (M; 8 kd), and an envelope protein (E; 53 kd) which is usually glycosylated (554). The M and E proteins are both associated with the lipid envelope by means of hydrophobic membrane anchors. The E protein is the major component of the virion surface; it contains the important antigenic determinants subserving hemagglutination-inhibition and neutralization and thus induces immunological responses in the infected host. Structural elements of the E protein determinants are assumed to be involved in the binding of virions to cell receptors and in intracndosomal fusion at low pH. These protein constituents are sensitive to enzymatic digestion with trypsin, chymotrypsin, and papain, which render the virus noninfectious but preserve certain antigenic reactivities, depending upon the degree of proteolysis.

Detergents and proteases have been used to characterize the structure of flaviviruses and to isolate immunologically reactive subunits (534,554). Nonionic detergents such as Triton-X solubilize the entire envelope, releasing M and E proteins; whereas sodium deoxycholate appears to remove only E, leaving M associated with the nucleocapsid. Protease treatment showed that a portion of the E glycoprotein is located within the lipid bilayer (215). Analysis of the primary structure of the flavivirus glycoprotein has confirmed the presence of a hydrophobic membrane anchor region at the carboxyl terminus of the E protein molecule (409).

The flaviviral envelope protects the genome from cellular nucleases, and naked nucleocapsids released by detergent treatment are degraded by ribonuclease. Flavivirus infectivity and hemagglutinin are optimally stable at pH 8.4 to 8.8 (262). Sensitivity to acid pH (also to bile and proteolytic and lipolytic enzymes) generally precludes infection by the oral route. Tick-borne encephalitis may, however, be acquired by ingestion of infected milk (431). Flaviviruses are rapidly inactivated at high temperature. At 50°C, 50% of infectivity is lost in 10 min. As a practical measure, total inactivation of virus suspended in blood or other protein solutions occurs within 30 min at 56°C. Low temperatures preserve infectivity, with stability being greatest at −60°C or below. Aerosols present a hazard of laboratory infection (498). St. Louis encephalitis virus is stable for 6 hr in acrosol suspension at room temperature and 23% to 80% humidity (262). For reasons that are not understood, aerosol laboratory infections with tick-borne flaviviruses are more frequent than with mosquito-borne viruses.

Flaviviruses are inactivated by ultraviolet light, gamma-irradiation, and disinfectants, including 3% to 8% formaldehyde, 2% glutaraldehyde, 2% to 3% hydrogen peroxide, 500 to 5,000 ppm available chlorine, alcohol, 1% iodine, and phenol iodophors. The tick-borne viruses appear to be relatively more resistant to these measures than mosquito-borne agents.

Antigenic Composition and Determinants

Antigenic Classification

All members of the genus *Flavivirus* share common antigenic sites, as revealed by hemagglutination-inhibition (HI) tests with polyclonal immune sera, and this originally formed the basis for their classification (73). Neutraliza-

tion tests (NT), on the other hand, are more discriminating and can be used to distinguish individual viruses in the genus and to define subgroups of closely related viruses.

On the basis of cross-neutralization using polyclonal hyperimmune antisera, the flaviviruses have been divided into a number of antigenic complexes. The original studies by deMadrid and Porterfield (106) classified 36 flaviviruses, and in a more recent analysis of 66 flaviviruses Calisher et al. (66) defined eight antigenic complexes encompassing 49 viruses (Table 2). Seventeen other viruses are not related closely enough to warrant inclusion in any of these complexes. Since the envelope protein E is the viral he-

TABLE 2. *Flavivirus antigenic complexes defined by close relationships in cross-neutralization tests with polyclonal antisera*[a]

Principal vector	Antigenic complex	Viruses[b]
Tick	Tick-borne encephalitis	(*Russian spring-summer encephalitis, Central European encephalitis), Omsk hemorrhagic fever, louping ill, Kyasanur forest disease. (Langat,* Phnom-Penh bat*, Carey Island*), *Negishi, Powassan,* Karshi, Royal Farm
	Tyuleniy	Tyuleniy, Saumaurez Reef, Meaban
Mosquito	Japanese encephalitis	*Japanese encephalitis, St. Louis encephalitis, Murray Valley encephalitis, West Nile, Kunjin, Usutu,* Koko-bera, Stratford, Alfuy, Kou-tango
	Ntaya	Ntaya, (Tembusu, Yokose), (*Israel turkey meningoencephalitis,* Bagaza)
	Uganda S	Uganda S, *Banzi,* Bouboui, Edge Hill
	Dengue	*Dengue 1, dengue 2, dengue 3, dengue 4*
None[c]	Rio Bravo	*Rio Bravo,* Entebbe bat, Dakar bat, Bukalusa bat, Apoi, Saboya
	Modoc	Modoc, Cowbone Ridge, Jutiapa, Sal Vieja, San Perlita

[a] The antigenic complexes correspond roughly to vector associations, implying evolutionary origin. Seventeen other flaviviruses are sufficiently distinct to preclude inclusion in these complexes.

[b] Parentheses indicate viruses more closely related to each other than to other members of the complex. Italics indicate viruses pathogenic for humans and/or domesticated animals.

[c] Viruses transmitted directly between vertebrate hosts, principally bats and rodents; *two members of the TBE complex may have this mode of spread.

Modified from Calisher et al. (66).

magglutinin and the primary target for neutralizing antibodies [reviewed in (213)], the cross-neutralization data reflect differences in the antigenic structure of this envelope protein and, together with the results of cross-HI tests, provide evidence for the presence of group-, serocomplex-, and type-specific determinants. This antigenic classification conforms to major biological and epidemiological characteristics of the flaviviruses (see Table 1).

The complexity of the antigenic relationships among flaviviruses has further been emphasized by the application of protein E-specific monoclonal antibodies [for review see (213,221,470)]. These analyses have demonstrated the presence of flavivirus group-, serocomplex-, and type-specific antigenic determinants, generally confirming the relationships shown with polyclonal antisera. Monoclonal antibodies have also been found with subcomplex, subtype, strain, and even substrain specificity, thus allowing the further distinction of viruses at a level that has been difficult or impossible to demonstrate with polyclonal antisera (22,501).

However, monoclonal antibodies have also uncovered antigenic relationships at the epitope level that link different flavivirus antigenic complexes or that reveal previously unsuspected relationships between members of the same antigenic complex, thus complicating the simple concept of group-, complex-, and type-specific determinants. For example, among members of the dengue complex, subcomplex specificities were demonstrated for dengue 1 and 4 and for dengue 2 and 3 (224). Relationships were also revealed between dengue and members of other antigenic complexes (e.g., Tembusu virus). Moreover, monoclonal antibodies may show functional reactivity with heterologous, but not homologous, virus strains. For example, monoclonal antibodies prepared against yellow fever 17D virus neutralized the parental Asibi strain but not 17D (501). To complicate matters even further, binding and functional assays with the same monoclonal antibody may show completely different cross-reactivity patterns (213,221). These data are an apparent reflection of the complexity of antigenic determinants recognized by antibodies on the surface of the virion.

In the last few years the complete or partial sequences of a number of flaviviruses have provided a classification based on sequence homologies and the construction of evolutionary trees (Fig. 2). Despite the considerable confusion introduced by the application of monoclonal antibodies, it is quite interesting to note that the amino acid sequence comparisons of protein E—like those of other viral proteins (38,321)—yield a picture that perfectly matches that of the flavivirus serocomplexes defined by cross-neutralization using polyclonal immune sera (Table 2).

The tick-borne encephalitis complex (TBE) and yellow fever virus are most distantly related to each other and to the other flaviviruses, with a sequence homology of 40% to 44% (depending on the pair of viruses compared). There is a somewhat closer relationship between members of the

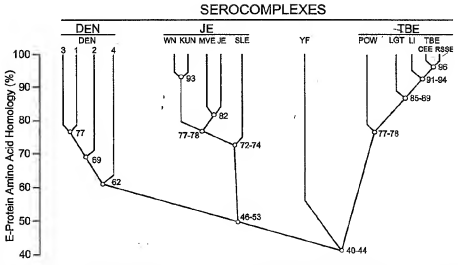


FIG. 2. Evolutionary tree of flaviviruses drawn on the basis of their envelope (E) protein amino acid homologies. These were calculated as percentage identical residues after optimized alignment of compared sequences counting gaps as mismatches using the Beckman Microgenie software package, Version 4.0. DEN, dengue; WN, West Nile; KUN, Kunjin; MVE, Murray Valley encephalitis; JE, Japanese encephalitis; SLE, St. Louis encephalitis; YF, yellow fever; POW, Powassan; LGT, Langat; LI, louping ill; TBE, tick-borne encephalitis. Modified from Mandl et al. (342) and Heinz et al. (221).

JE serocomplex and the dengue viruses (46% to 53%). The different dengue types share 62% to 77% of their amino acids, with dengue 1 and dengue 3 being most closely related (77% homology), followed by dengue 2 (69%) and dengue 4 (62%). An extensive comparative sequence analysis of dengue 2 virus isolates has revealed a significant degree of diversity (up to 10% amino acid differences in protein E) and has allowed the identification of at least four genetic subtypes (176,321,463). Within the JE and TBE serocomplexes the degree of nucleotide homology is at least 72% and 77%, respectively. As pointed out by Blok et al. (38), the viral proteins are very similar in topology. There is no evidence of genetic recombination, suggesting that flaviviruses have evolved by divergent mutational change.

It is of interest to note that protein E is one of the most slowly evolving viral proteins (together with NS1, NS3, and NS5), indicating that immunologic pressure is not an important driving force in the evolution of flaviviruses.

Role of Structural and Nonstructural Proteins in Immune Response

Being the major component of the virion surface, the envelope protein E plays a dominant role in the generation of neutralizing antibodies and the induction of a protective immune response. This has been conclusively demonstrated by active immunization of animals with defined subviral components (221) and recombinant proteins (49,253,278, 280,352,368,428,507) and by passive protection experiments with protein E-specific monoclonal antibodies [reviewed in (213,470)]. Protein E-specific nonneutralizing antibodies or neutralizing antibodies at subneutralizing concentrations, however, may also mediate the phenomenon of antibody-dependent enhancement (ADE) of infection, which has been implicated in the pathogenesis of dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (190).

Low titer neutralizing activity and a significant degree of passive mouse protection has also been observed with monoclonal antibodies against the prM protein of dengue 3 and dengue 4 virus (264). Mice immunized actively with prM/M also exhibit a degree of protection (47). The prM protein is part of immature virions, and its proteolytic cleavage, presumably by a cellular protease in the trans-Golgi network, generates mature virions. In certain instances this precursor cleavage can apparently be incomplete, thus allowing the prM protein to function as an additional target for neutralizing and protective antibodies.

The nonstructural glycoprotein NS1 is expressed on the surface of infected cells (69,132,504,532) but is also secreted and in this form was previously designated as *soluble complement fixing* (SCF) antigen (46,133,534). NS1 elicits an immune response in the course of flavivirus infections in humans (290) and in experimental animals (205). Its role as a protective antigen is discussed below. In the infected host, secreted forms of NS1 are found in the circulation.

Although passive protection in mice by a monoclonal antibody against the nonstructural protein NS3 of dengue 1 virus has been reported (542), the primary immunological role of the nonstructural proteins, with the exception of NS1, seem to be their function as targets for cytotoxic T cells (Tc) (230,486).

The Antigenic Structure of Protein E

Protein E is the primary target for neutralization, and its antigenic structure has been extensively studied using both polyclonal and monoclonal antibodies [for review see (213, 221,470)]. Since the location of disulfide bridges is also known (409), these data together have been used to generate structural models of protein E (68,219,341,469,471).

Recently the x-ray structure of a soluble form of the TBE virus protein E was determined (457). This soluble form was obtained by tryptic cleavage from purified virions (220) and lacked the carboxy-terminal 80 to 90 amino acids including the membrane anchor. Since the cysteines forming all six disulfide bridges are absolutely conserved among flavivirus E proteins and a common 3D structure can be anticipated, the available data on other flavivirus epitopes will be discussed with reference to the TBE virus protein E.

Unlike the influenza hemagglutinin, this complex is a head-to-tail rather than a head-to-head oligomer. Thus, it apparently does not represent a spike protruding from the surface of the virion, but rather a 170-Å-long rod that is probably anchored to the membrane at its distal ends. The side view shows that the dimer has a curved shape consistent with its attachment on the surface of a 500-Å particle. In each monomer three structural entities are discernable, two of which are related to previously defined antigenic domains [compare Fig. 3A and (341)]. The carboxy-terminal 100 amino acids (domain III in Fig. 3A) correspond to antigenic domain B and form a β-barrel composed of seven antiparallel β-strands resembling an immunoglobulin constant domain. This structure is connected by a flexible region to the central domain I, which is folded as an eight-stranded β-barrel with up-and-down topology. It includes the amino terminal 50 amino acids as well as a sequence element that carries the single carbohydrate side chain and was previously referred to as *antigenic domain C*. Two long loops extending from this central part of the protein are primarily involved in dimer contacts (domain II in Fig. 3A) and correspond to antigenic domain A.

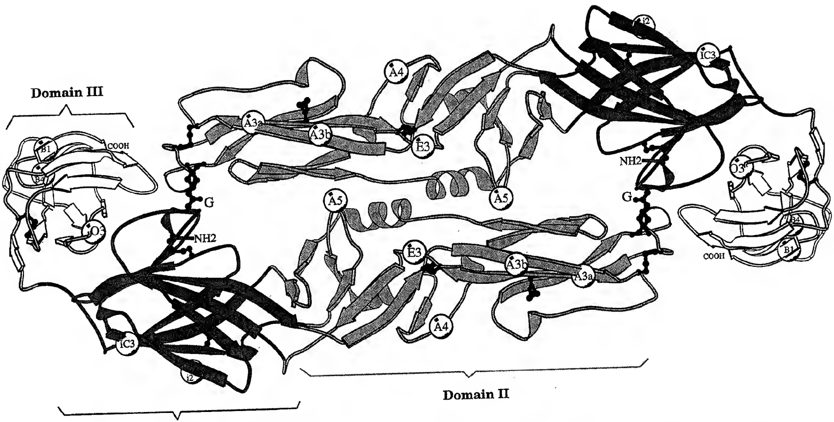
Linear epitopes have been mapped using synthetic peptides and are found in areas of the glycoprotein predicted to be hydrophilic (246); however, the induction of neutralizing antibodies seems to be strongly dependent on the native conformation of protein E (584). The location of neutralization sites can be inferred from the position of amino acid substitutions in monoclonal antibody-escape mutants. Mutations leading to escape from neutralization have been mapped to each of the structural domains shown in Fig. 3A (74,148,205,238,256,322,341), and the position of such amino acid substitutions in TBE virus mutants are indicated in Fig. 3. Consistent with the suggested topology of the dimer on the virion surface, most of these mutations are located on the accessible upper side of the subunit, which also suggests that they indeed form part of the corresponding epitopes. In contrast, two of the mutations in TBE virus (B1 and B4) are not located on top but on the side of domain III, and may therefore alter the conformation rather than being part of the epitope itself. Interestingly, one of the mutations (designated *O3*) does not affect binding of the selecting monoclonal antibody to the virus but nevertheless abolishes its neutralizing activity. The scattered distribution of these neutralization-escape mutations over the entire subunit indicates that antibody binding to any of the structural domains can lead to virus neutralization. Taking into account the known sizes of Fab footprints on protein antigens which are in the range of 500 to 900 Å^2 and involve 15 to 20 contact residues (104,520,575), most of the epitopes are expected to be discontinuous. This is consistent with the denaturation sensitivity of neutralization epitopes (584) and the finding that monoclonal antibodies usually do not react with synthetic peptides unless they recognize an epitope that is present on denatured protein E only (183,238,471).

Antigenic domain B (domain III in Fig. 3A) represents an independently folding protein domain that can be isolated as a trypsin-resistant fragment from purified virions (594) or generated as a recombinant protein by bacterial expression (349). In both cases, however, its reactivity with neutralizing monoclonal antibodies is dependent on the integrity of the single disulfide bridge located within this domain.

Since the dimeric subunit forms part of an as yet undefined lattice on the virion surface, it is likely that certain epitopes are composed of elements from different subunits. Although formal proof is lacking, some neutralization epitopes present on whole virions are lacking on the soluble dimer (220) and on the Triton X-100 solubilized protein.

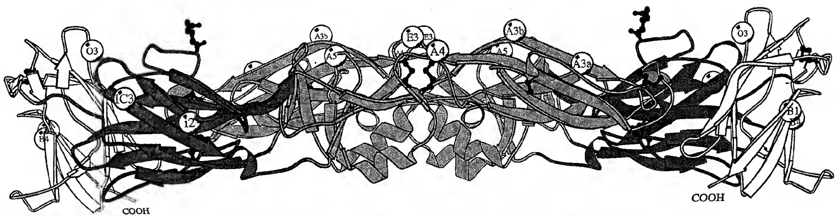
The mechanisms of neutralization by these antibodies remains a matter of speculation. The functional sites of protein E have not yet been mapped, though it has been speculated that domain III may be involved in receptor binding (329) and that the highly conserved sequence from residues 98 to 111 at the tip of one of the domain II loops (Fig. 3A) may be involved in fusion activity (472). Fusion activity requires conformational changes that affect several neutralization epitopes, primarily within the central domain I and domain II (222,472). These changes are apparently associated with a reorganization of the subunit interactions on the virion surface, with trimer contacts being favored in the low pH form, as opposed to dimer contacts in the native form (7). It can be assumed that the interference with these structural rearrangements by antibody binding represents one mechanism leading to virus neutralization.

Hemagglutination-inhibition and NT activity are not necessarily linked, and all possible combinations (HI+, NT+; HI-, NT+; HI+, NT-) have been observed with monoclonal antibodies [reviewed in (213)]. It was also observed that binding of certain monoclonal antibodies was enhanced in the presence of other antibodies (218,226). Such cooperative effects were not only demonstrated in binding assays but also in neutralization and passive protection ex-



Domain III
COOH
B1
B4
O3
G
NH2
IC3
i2
A3a
A3b
A4
E3
A5
Domain II

A



O3
IC3
i2
B4
COOH
A3b
A5
E3
A4
A3a
B1

B

periments, even with antibodies that were nonfunctional when tested alone.

In several instances, monoclonal antibodies were also identified that did not neutralize the virus but were protective in passive immunization experiments (45,162). Such nonneutralizing antibodies may even confer cross-protection between different flaviviruses. For example, a group-reactive nonneutralizing dengue antibody (4G2) provides efficient protection against yellow fever challenge (43). The ability of nonneutralizing antibodies to protect was not associated with complement-dependent lysis of infected cells, but a possible mechanism of protection is suggested by the finding that the protective antibodies inhibited virus replication in a neural cell line, although they showed no neutralization in Vero cells.

The Antigenic Structure of NS1

By the use of polyclonal and monoclonal antibodies, type-specific, sub-complex specific, complex-specific, and flavivirus-crossreactive epitopes have been identified (131), and a detailed epitope map of the secreted form of dengue 2 NS1 was established (225,604). Similar to the situation with protein E, most of the epitopes are conformation-dependent and only in rare cases reactivities with linear epitopes were observed (131). Oligomerization is a general property of flavivirus NS1 proteins. The formation of dimers seems to be specifically favored in certain instances (595) and seems to be necessary for secretion (441).

Although antibodies to NS1 do not react with the virion and exhibit no neutralizing activity, passive transfer of some monoclonal antibodies conferred protection against dengue and yellow fever virus infection in experimental animals (162,227,502). Enhanced protective efficacy was observed with combinations of monoclonal antibodies (227), suggesting that protection might be due to immunological recognition and destruction of infected cells expressing NS1 (507). This phenomenon seems to be dependent on the Fc portion of antibodies (506) and could be due to complement-mediated cytotoxicity. However, other mechanisms may contribute as well, since protection has also been observed in the absence of complement fixation (227).

Since the phenomenon of antibody-dependent enhancement of infection by antivirion antibodies has been implicated in the development of DHF/DSS, NS1 has been evaluated as a candidate vaccine for dengue and other flaviviruses (45,605,608). Active immunization experiments using NS1 purified from infected cells or recombinant NS1 demonstrated the induction of a protective immune response in experimental animals against homologous challenge with dengue, yellow fever, or TBE virus (252,502, 503). The specific immunogenicity apparently depends on the physical structure of the NS1 preparation. Dimers were superior to monomers (131), and higher protection rates were observed with the soluble NS1, compared to its membrane-associated form.

Specificity and Targets for Cellular Immune Responses

The specificity of T-cell responses to flaviviruses both in the human and mouse systems has been most extensively studied with members of the dengue serocomplex and the JE serocomplex (WN, Kunjin, MVE) (230,305,567). In the course of dengue virus infection of humans, both CD4+, CD8- as well CD8+, CD4- T-lymphocyte responses have been detected and characterized. In bulk cultures of CD4+ lymphocytes as well as with CD4+ T-cell clones obtained from a dengue virusinfected individual, different specific cross-reactivity patterns with other dengue viruses, WN, and yellow fever virus were observed (299,611). Interestingly, these CD4+/CD8- T-cell clones were cytotoxic and exhibited a class II MHC-restricted cytotoxic activity against infected lymphoblastoid cell lines.

Similar results were also obtained with CD8+ T lymphocytes from infected humans and mice. Bulk responses and those of individual T-cell clones were cross-reactive with other dengue serotypes (57,486), supporting the concept put forward by Kurane and Ennis (305), that the activation of memory T cells during secondary infections with a heterologous serotype may contribute to the development of DHF/DSS (see below). In this context it is of interest to note that the induction of cross-reactive T cells seems to differ in individuals with different genetic backgrounds. A predominantly type-specific CD4+ T-lymphocyte response was observed in an individual vaccinated with an experimental live-attenuated dengue virus type 1 vaccine (166) and the extent to which WN-immune CD8+ cells as well as CD4+ T cells from mice recognized Kunjin-virus-encoded determinants varied considerably between mice of different MHC haplotypes (231,286).

Protein targets for T-cell responses were identified both in humans and in mice. In the dengue virus system, NS3 appears to contain multiple (at least six) dominant epitopes for CD4+ and CD8+ T lymphocytes of both species [reviewed by Kurane and Ennis (305)], but T-cell epitopes were also recognized on E and NS1. Epitopes on NS3 were

FIG. 3. Ribbon diagram representing the structure of the membrane anchor-free envelope (*E*) protein dimer isolated from tick-borne encephalitis virus. **A:** Top view. **B:** Side view. The positions of amino acid substitutions in monoclonal antibody escape mutants are indicated by white circles and designated according to Mandl et al. (341) and Holzmann et al. (in preparation). Disulfide bridges and the single carbohydrate side chain (*G*) attached to domain I are shown as ball-and-stick representations.

found to have serotype, subgroup, and flavivirus group specificities. One epitope recognized by a dengue-specific human CD4+ T-cell clone was mapped to a peptide including amino acids 255 to 264 of NS3, which is conserved among all four dengue types (300).

NS3, NS4A, and NS4B were shown to be predominant targets for MHC I-restricted anti-Kunjin, -WN and -MVE responses in mice (231,326); whereas MHC II-restricted responses to WN and Kunjin virus were predominantly directed against determinants on structural proteins and/or NS1 (286). In any case, a very limited number of T-cell determinants seems to be recognized by a particular individual, and variation exists between different haplotypes.

Potential T-cell epitopes in protein E of MVE, JE, WN, and dengue virus were identified by the use of computer predictions and by analyzing *in vitro* T-cell proliferation responses against synthetic peptides (306,313,471). Functional identification of T-helper cell epitopes in protein E was achieved by measuring the B-cell response after immunization with synthetic peptides derived from MVE virus (354,473) and dengue 2 virus (474). T-cell help and enhanced antibody response was dependent on the linkage between the corresponding peptides, and the most efficient T-B cell epitope interaction occurred when the peptides were colinearly synthesized.

Antibody-Dependent Enhancement

Antibody-dependent enhancement of flavivirus replication in Fc-receptor-bearing peripheral blood monocytes and macrophage-like cell lines has been demonstrated *in vitro* with a number of flaviviruses, including dengue, yellow fever, Wesselsbrop, WN, TBE viruses (193). Increased adsorption of virus particles to host-cell plasma membrane (mediated by subneutralizing antibodies), as well as increased efficiency of virion internalization has been shown to increase viral replication five- to sixfold in monocyte- or macrophage-like cells (156,157).

Antibody-dependent enhancement has been demonstrated to occur via FcγRI and FcγRII (281,323), but a second type of enhancement dependent upon the C3 complement receptor has also been described (70). Attempts with monoclonal antibodies to elucidate whether specific epitopes are responsible for ADE have revealed that: (a) there is no correlation between serological specificity and ADE, i.e., it can be mediated by broadly cross-reactive as well as type- and even subtype-specific antibodies; and (b) antibodies with different functional activities (i.e., neutralizing, hemagglutination-inhibiting or nonfunctional antibodies) can mediate ADE. Studies with a number of dengue 2 and dengue 4 strains and a panel of monoclonal antibodies has revealed significant heterogeneity and strain-specific patterns of ADE reactivities has been discerned (195,393). The possible role of ADE in the immunopathogenesis of DHF is discussed below.

Molecular Basis of Virulence

Strain-specific differences in neurovirulence and/or neuroinvasiveness have been observed with several flaviviruses (22,23,111,240,241,382). Attenuation by serial passaging formed the basis for the development of the yellow fever 17D vaccine (546) and candidate vaccine strains for JE (408,606) and dengue virus (602). Attenuation during serial passage may occur quite rapidly. For example, the vaccine strain of dengue type 1 developed in Thailand by Bhamarapravati et al. differs from its parent (#16007) by 39 nucleotide and 18 amino acid changes after only 13 passages in primary dog kidney cells (P.W. Mason, personal communication, 1994), and mosquito-borne flaviviruses become rapidly attenuated after only 6 passages in HeLa cells (24,124,212). These observations are best explained in part by the high rate of mutation of RNA viruses (24,236) and fixation of specific mutations during passage in a specific and homogeneous cell type.

Nucleotide sequencing of pairs of parental and attenuated vaccine derivatives has provided some clues as to the molecular basis of virulence, but the large number of mutations and their localization in many parts of the genome complicate interpretation of these comparative data (38, 187,408,461). The number of nucleotide changes separating parental and vaccine strains is 67 for yellow fever virus (see below); 53 for dengue type 2 virus (38); 39 for dengue type 1 virus (P.W. Mason, personal communication, 1994); and 45 for JE virus (408). In the case of yellow fever, although 67 nucleotide and 31 amino acid differences distributed across the genome were originally noted between 17D-204 vaccine and the parental Asibi virus (187), subsequent analyses of other vaccine strains derived from the 17D lineage and additional wild-type yellow fever viruses have significantly reduced the mutations that may explain attenuation (254,496). Sequencing of the vaccine substrains 17D-204, 17DD and 17D-213, and comparison with Asibi showed that only 50 nucleotide changes and 13 nonconservative amino acid substitutions were specific to the vaccine strains, 5 of which occur in the E gene. Since the 5 changes in the E gene occur at sites (amino acids 52, 173, 200, 305, and 380) that are conserved in virulent yellow fever viruses from both Africa (254) and South America (18) isolated many years apart, these mutations are likely to be implicated in attenuation.

The location of these mutations with respect to functional domains of the E gene lend further support to this conclusion (compare Fig. 3A). Two of the mutated amino acids (at positions 52 and 200) are present in the fusion sequence in domain II (341). Two changes (at amino acids 305 and 380) are present in domain III, and one (at amino acid 173) in domain I of the E protein. The mutations at positions 173 and 380 are very close to neutralization or virulence determinants mapped by monoclonal antibody escape mutants of TBE virus (237,341). Studies of envelope proteins of yellow fever viruses with monoclonal an-

tibodies supports the notion that antigenic changes in the E protein are implicated in attenuation or virulence (24). In the case of the NS1 gene, a 17D-specific mutation at the N-terminus of NS1 is similar to a change observed between parental and vaccine strains of dengue 2 virus (38). The 17D-specific alteration in NS3 yields a nonconservative amino acid change (D→E) in the C-terminal portion of the protein involved in linking the presumed helicase and RNA triphosphatase functions (585). In summary, a strong rationale exists implicating one or more of seven nonconservative changes in E, NS1, or NS3 in attenuation of yellow fever vaccine, a hypothesis that can now be empirically tested using site-specific mutations of an infectious cDNA clone (460).

Yellow fever virus exhibits two distinct virulence factors reflecting its capability to induce encephalitis (neurotropism) and hepatitis (viscerotropism). The attenuated 17D vaccine virus has lost its capacity to cause hepatitis while retaining a reduced degree of neurotropism, particularly for the immature brain. A single fatal case of human encephalitis due to 17D virus has been reported (10), and the virus recovered from the brain was subjected to careful scrutiny (255). A monoclonal antibody specific for wild-type yellow fever virus recognized the case strain but not the parental vaccine (17D-204 substrain) and was considered a mutant at this epitope. The case strain also differed phenotypically in being neuroinvasive and neurovirulent for mice inoculated intranasally and appeared to be neurovirulent for monkeys inoculated intracerebrally. Sequencing of the E glycoprotein and comparison with other substrains of 17D vaccines revealed a single amino acid change (K→Q) at position 303 that could be correlated with the increased neurovirulence of the virus recovered from brain tissue. Presumably this mutation arose during replication of the vaccine in the human host. Interestingly, this mutation is spatially close to two other mutations distingishing wild-type from vaccine virus (including the nonconservative change at amino acid 305), suggesting that this region of domain III may represent an important locus defining neurovirulence.

Other data on the localization of virulence determinants have come from characterization of monoclonal antibody-escape mutants (74,237,256,327), host range mutants (329), virulence revertants (254), and by the engineering of infectious cDNA clones derived from dengue 4 virus and JE virus (541). These studies demonstrate that the E protein plays a dominant role as a determinant of flavivirus virulence. Single amino acid substitutions in the E protein can have dramatic effects on virulence. A chimeric dengue 4 virus containing the envelope proteins from TBE virus exhibited the neurovirulence phenotype of TBE virus (429). In contrast to TBE virus, however, this chimera was not neuroinvasive, indicating that other regions in the genome contribute to the virulence properties of this virus.

Specific virulence mutations in protein E map to distinct structural elements (compare Fig. 3A). Domain III (C-terminal in the crystallized membrane anchor-free fragment), the element that links the central domain I with the loops of domain II, the tip of domain II, and the carbohydrate side chain. The importance of domain III was directly demonstrated by the analysis of single amino acid mutants of MVE, TBE, louping ill, and JE viruses (74,237,256,329). Amino acid substitutions in the E protein of the yellow fever 17D vaccine strains also cluster within this relatively small domain (187,496). Since this domain contains an RGD sequence in several mosquito-borne flaviviruses, it has been proposed to form part of the receptor-binding site, and this could explain its role in virulence (329). A mutation in the RGD sequence of MVE virus led to loss of virulence for mice (330).

Specific mutations within the element linking the central domain I with the domain II loops were shown to influence virulence with TBE (Holzmann H et al., in preparation), JE (74,205), and MVE viruses. The latter case is especially interesting since the attenuating mutation also lowered the pH threshold and the extent of fusion, suggesting a relationship between fusion activity and virulence (McMinn PC, personal communication, 1994).

Using the TBE-dengue 4 virus chimera, Pletnev et al. (430) engineered specific mutations in the conserved sequence element at the tip of domain II, which led to an increase in neurovirulence. This sequence interacts with domain III of the other monomer and thus forms an important contact point within the dimeric subunit. These interactions may also involve the juxtaposed carbohydrate side chain, the removal of which was shown to either reduce (in the case of the TBE-dengue 4 chimera) or even enhance neurovirulence (in the case of a dengue 4- dengue 4 chimera) (265). As demonstrated with the TBE-dengue 4 chimera (430), such effects of carbohydrate side chains on virulence are not restricted to protein E, but apply to the prM protein and NS1 as well. Also, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera, consistent with the importance of prM cleavage to yield fully infectious virus (222,583).

Other regions of the flavivirus genome have not yet been precisely mapped with respect to virulence determinants, but much more information is expected to become available soon by the further extensive application of the infectious clone technology.

Virus-Cell Interactions

Host-cell macromolecular synthesis is not markedly decreased until late in flavivirus infection, when cytopathic effects (CPE) appear. As discussed further below, many arthropod and vertebrate cultured cells do not exhibit CPE. In other cell-virus pairings, infection progresses to CPE within an interval as short as 18 hr or as long as 5 to 7 days. A major change in infected cells is proliferation and hypertrophy of rough endoplasmic reticular membranes with-

in which virus particles accumulate. At the ultramicroscopic level, cellular pathologic changes include mitochondrial damage, fragmentation of reticular membranes, formation of distended vacuoles and inclusion bodies, increase in lysosomal bodies, and rarefaction of cytoplasm (398). Activities of lysosomal enzymes increase in infected tissues. At the microscopic level, susceptible vertebrate cells such a HeLa, BHK-21, porcine kidney (PS), and primary chick or duck embryo display cell rounding, shrinkage, pyknosis of nuclei, and dislodgement from the growth surface (Fig. 4).

Cell fusion and syncytium formation (polykaryocytosis) have been observed in mammalian and arthropod cells. Fusion and infectivity are inhibitable with ammonium chloride, typical of viruses that enter cells by receptor-mediated endocytosis and low-pH mediated membrane fusion in lysosomes. Fusion from without is demonstrable in mosquito cells after exposure of cell-bound virus to low pH (184,449).

Unlike vertebrate cells, arthropod cells infected with some flaviviruses are capable of massive proliferation and hypertrophy of cytoplasmic membranes, as well as very great production of virus, often without sustaining cytopathologic changes. In contrast to mosquito cell cultures, those derived from ticks do not demonstrate CPE after infection with tick-borne flaviviruses.

In general, mosquito-borne viruses replicate in mosquito cell culture, and a few (such as SLE and WN, which have been isolated from ticks in nature) will also grow in tick cell cultures. Tick-borne flaviviruses replicate in tick cell cultures, but not consistently in mosquito cells. The antigenic reactivity of some flaviviruses is altered by replication in mosquito cells. For example, dengue and Kunjin viruses lose hemagglutinating activity on passage in *Aedes albopictus* cells (405).

Infected cells stained by the fluorescent antibody technique show viral antigen expression in cytoplasm, typically with a perinuclear concentration of reactivity. Several investigators have also described nuclear fluorescence in cells infected by JE virus. This reactivity does not reflect a nuclear phase of virus replication, but rather cross-reactivity with host-cell nuclear histones.

Persistent noncytopathic infections by flaviviruses have been induced in a variety of arthropod and vertebrate cell lines. Depending on the virus-cell pairing, such infections can involve few cells in the culture (508) or nearly 100% of the cells (53). Progeny virus from persistently infected cultures often exhibit altered antigenic reactivity, reduced virulence, temperature sensitivity, or other signs of mutation. The generation of defective interfering particles appears to be an important mechanism underlying persistence (435,508), and interferon production does not play an important role.

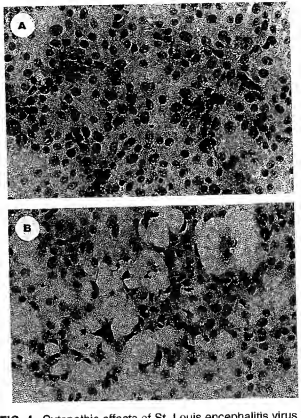


FIG. 4. Cytopathic effects of St. Louis encephalitis virus in Vero cell culture. **A:** Normal culture, Mayer's hematoxylin, ×220. **B:** Culture after 5 days incubation, showing focal cytopathic effects, shrinkage, and rounding of cells, pyknotic nuclei, and appearance of hole in the cell monolayer.

Propagation and Assay in Cell Culture

Flaviviruses produce CPE and plaque formation in a variety of primary and continuous cell cultures derived from human, monkey, rodent, swine, and avian tissues [reviewed in (262)]. Cell cultures of reptilian, amphibian, and arthropod origin also support replication with or without CPE or plaques. Virus yields and titers, grade of CPE, plaque size and quality, and rate of growth vary with the specific virus and host cell (see discussion of individual viruses). In general, the BHK-21 (baby hamster kidney), SW-13 (human adrenal carcinoma), PS, *Aedes* mosquito cells, and primary chick and duck embryo cells produce the highest virus yields, in the range of 9 to 10 dex/mL. In comparison with alphaviruses, the latent period of flavivirus growth is relatively long, and growth is slow. At high multiplicity of infection in BHK-21 cells, SLE virus has a latent period of 11 hr and reaches peak titer at 28 hr. Plaques appear after 4 to 6 days; double overlay procedures are often required for optimal plaque assays in mammalian cell cultures. Addition of neutral red to agar overlay may enhance plaque size. Assay of dengue virus infectivity requires special techniques (see below).

In addition to observation of CPE or plaques, flavivirus growth in cell culture may be measured by immunofluorescent staining or detection of antigen in supernatant fluids by immunoassay, complement fixation (CF), hemagglutination, or polymerase chain reaction (PCR). In the case of arthropod cells that do not produce CPE or plaques, these techniques are mandatory, or supernatant fluid must be passed to a susceptible host (mice or mammalian cell culture).

Infection in Experimental Animals; Host Range

The susceptibility of experimental animals and host range vary widely and are considered in the discussion of individual viruses.

Newborn mice and hamsters inoculated intracerebrally are somewhat more sensitive for infectivity assay and primary isolation of many flaviviruses than are cell cultures; the slight advantage in sensitivity is, however, offset by the expense and inefficiency of *in vivo* assays. For other flaviviruses, including yellow fever and dengue, cell cultures provide more sensitive assay systems. In general, intrathoracic or intracerebral inoculation of live mosquito adults or larvae provides the most sensitive system for assay of mosquito-borne flaviviruses.

PATHOGENESIS AND PATHOLOGY

The greatest body of information about flavivirus pathogenesis is derived from experiments on mice and other laboratory rodents. These animals provide a reasonably good model of flavivirus encephalitis but not of other syndromes associated with human flavivirus infection (i.e., fever, arthralgia, rash, and hemorrhagic fever). Viruses that produce these syndromes in humans, including dengue and yellow fever, cause encephalitic infections in laboratory rodents. The pathogenesis of dengue and yellow fever is discussed in their respective sections. The universal neurotropism of flaviviruses in rodents and even in arthropod vectors (in which brain and ganglia are major sites of replication) may reflect evolutionary conservation of viral polypeptide structures involved in receptor interactions and of cell membrane molecules which subserve virus-receptor interactions.

Three patterns of pathogenesis have been described in flaviviral encephalitis [for review, see (259,379,402): (a) fatal encephalitis, usually preceded by early viremia and extensive extraneural replication; (b) subclinical encephalitis, usually preceded by low viremia, late establishment of brain infection, and clearance with minimal destructive pathology; and (c) inapparent infection, characterized by trace viremia, limited extraneural replication, and no neuroinvasion.

Virus and Host-Specified Factors Influencing Pathogenesis

The course and outcome of infection is influenced by both virus- and host-specified factors. High dose and intracerebral or intranasal routes of virus infection predispose to fatal encephalitis. Virus strains may differ in neuroinvasiveness and/or neurovirulence.

Among host factors influencing pathogenesis, the most important are age, sex, genetic susceptibility, and preexisting infection or immunity to heterologous agents. Neonatal animals are more susceptible to lethal encephalitis than oldcr animals. Neonatal animals inoculated by the peripheral route are susceptible until 3 to 4 weeks of age, when resistance develops, but they may remain susceptible to lethal encephalitis when inoculated intracerebrally. Immature neurons have been shown to be more susceptible to infection than mature neurons (412). Parallels are seen in some human flavivirus infections. For example, young infants are more susceptible to encephalitis following yellow fever vaccination. In the case of some flaviviruses (e.g., SLE), however, susceptibility to encephalitis increases with advancing age, with the elderly being most severely affected. The mechanisms underlying the increasing susceptibility with age are not known, but they may include age-related waning of immunological responsiveness and the presence of underlying diseases that impair immune function or reduce the effectiveness of the blood-brain barrier. Physiological factors that lead to transient immunosuppression may also be responsible for increased susceptibility to flavivirus encephalitis. For example, mice exposed to cold or isolation stress and challenged with WN virus demonstrated higher virus replication in peripheral tissues and brain, and higher mortality rates (27).

Sexually mature female mice demonstrate increased resistance to some flavivirus infections (8). Sex differences in susceptibility of humans (as opposed to exposure to infected vectors) have not been demonstrated, except possibly in the case of DHF, which preferentially affects female children.

Genetic determinants play a central role in the pathogenesis of flavivirus infections. Studies by a number of workers (371,490,581) have demonstrated genetic resistance of nonimmune mice to flavivirus infection in a number of inbred mouse strains and showed that resistance was determined by a single autosomal dominant allele, currently designated as *Flv*'. The resistance allele was incorporated into susceptible C3H/He inbred mice to yield the resistant strain C3H/RV (C3H.PRI-*Flv*'). West Nile virus yields in brains of resistant C3H.PRI-*Flv* mice were significantly lower than in susceptible mice, but there were no differences in interferon or humoral antibody responsiveness between the two mouse strains (159). *In vitro* studies with cells derived from the two mouse strains indicated that (a) macrophages from resistant mice do not support flavivirus replication as well as macrophages from the sus-

ceptible strain and (b) the lower virus yields observed in cells of resistant mice were due to greater production of defective interfering virus particles (100). In the case of Banzi virus infection of C3H.PRI-*Flv* mice, genetic resistance did not appear to depend on lack of permissiveness of tissues to virus replication (250) but, instead, had an immunological basis (34,251). Virologic mechanisms may also operate in determining resistance in this model, however. Increased interfering virus found in lymphoid tissues of resistant mice may contribute to survival (517).

Studies in inbred mice derived from single subspecies of wild *Mus musculus* suggest that flavivirus resistance genes are carried by many wild mouse strains. In addition to *Flv'*, a second autosomal dominant allele at the *Flv* locus (designated Flv^{mr}) was found to determine resistance. Although not fully mapped, the *Flv* locus is believed to reside on chromosome 5 (495). Haplotype restriction may also influence the immune response of the host to flaviviruses. Inbred mouse strains of the $H\text{-}2^q$, $H\text{-}2^k$, and $H\text{-}2^b$ haplotypes differed with respect to neutralizing antibody response to JE vaccine virus strains (593).

Concurrent infections with unrelated agents may enhance flavivirus neuroinvasion [reviewed in (379)], presumably by disturbing the blood-brain barrier. This mechanism has been reported in mice doubly infected with JE, herpesvirus, *Trichinella*, and visceral larva migrans; a similar phenomenon has been reported to occur in humans with neurocysticercosis (102,511). Impairment of the blood-brain barrier by needle puncture, hypercarbia, microwave radiation, or administration of bacterial lipopolysaccharide or sodium dodecylsulphate renders mice susceptible to lethal encephalitis by virus strains that are inherently neurovirulent but not neuroinvasive (276,312,336).

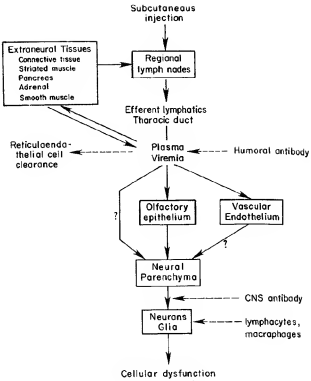


FIG. 5 General scheme of the sequential development of flavivirus infection. Boxes indicate sites of virus replication and dashed arrows show immune defense mechanisms. Modified from Nathanson (402), with permission.

Extraneural Infection and Routes of Neuroinvasion

A general scheme for the dissemination of flaviviruses in the host is shown in Fig. 5. After inoculation into the skin, the virus replicates in local tissues and regional lymph nodes. Virus is then carried via lymphatics to the thoracic duct and into the bloodstream (340). This primary viremia seeds extraneural tissues, which, in turn, support further viral replication and serve as a source for release of virus into the circulation.

The viremia level is modulated by the rate of clearance by macrophages, and it is terminated by the appearance of humoral antibodies, usually by approximately 1 week after infection. Major extraneural sites of flavivirus replication include connective tissue, skeletal muscle and myocardium, smooth muscle, lymphoreticular tissues, and endocrine and exocrine glands. In baby hamsters infected with SLE and Rocio viruses, pancreas and heart were the most severely affected organs (202). Virus particles within secretory granules of exocrine and endocrine areas of the pancreas were released by exocytosis. Myocardial necrosis with productive viral infection of myocytes was a prominent finding. Correlations are possible between this experimental model and pathogenesis in clinical hosts. Infection of goat mammary glands, followed by secretion of virus in goat's milk, is an important mode of spread of TBE viruses. Interstitial myocarditis has been reported in WN and JE of humans and horses, and pancreatitis has been associated with human WN virus infection.

Investigation of experimental flavivirus encephalitis in mice has demonstrated a relationship between level of viremia, development of brain infection, and multisite appearance of viral antigen in nervous tissue (5), supporting the concept of hematogenous spread to the central nervous system (CNS) (259). As pointed out above, artificial disruption of the blood-brain barrier potentiates neuroinvasion and encephalitis. The mechanism by which flavivirus particles cross the blood-brain barrier during natural infection remains uncertain. The ability of these viruses to replicate in vascular endothelial cells suggests that they may "grow across" capillaries in the brain. However, viral antigen has been found only rarely in endothelial cells of brain capillaries *in vivo*, and flaviviruses did not replicate to high titer in brain endothelium cells *in vitro* (123).

The olfactory tract has long been recognized as (a) an alternative pathway to the CNS and (b) an important mode of spread following aerosol exposure. Intranasal inoculation of flaviviruses may result in lethal encephalitis, presumably by direct infection of olfactory neurons and spread via the olfactory tract to the brain, whereas peripheral inoculation of the same virus strains does not result in neuroinvasion. However, olfactory neurons may also provide a route for CNS infection after peripheral virus infection. In an experimental model, mice and hamsters inoculated by the peripheral route with SLE virus developed low-level or undetectable viremias similar to those occurring in clinical hosts. This resulted in early infection of olfactory neurons (which are unprotected by blood-brain barrier) and subsequent axonal transport of virions to the olfactory lobe of the brain (383). It is not known whether this pathway operates in humans, but a postmortem study of JE patients indicated the hematogenous, rather than the olfactory, route of neuroinvasion (260). Once in the CNS, virus spreads rapidly.

Neuronal centers vary in susceptibility. In the mouse the hippocampal formation is particularly sensitive, whereas in monkeys and humans the thalamus, substantia nigra, and cerebellum are most vulnerable (401,402).

Pathological Changes

Pathological changes observed in humans and experimental animals with flaviviral encephalitis include: (a) neuronal and glial damage caused directly by viral injury and characterized by central chromatolysis, cytoplasmic eosinophilia and cell shrinkage, and neuronophagia; (b) inflammation, including perivascular infiltration of small lymphocytes, plasma cells, and macrophages (Fig. 6); (c) cellular nodule formation composed of activated microglia and mononuclear cells (Fig. 7); and (d) cerebral interstitial edema. In monkeys and hamsters infected with tickborne viruses, astrocyte proliferation and hypertrophy appear as a late phenomenon (613). At the ultrastructural level, infection of neurons is characterized by marked proliferation and hypertrophy of rough endoplasmic reticulum (RER), accumulation of vesicular structures derived from the RER and containing virus particles, and progressive degeneration of the RER and Golgi apparatus (203). Neuronal destruction is infrequent, however, suggesting that dysfunction rather than cell death is responsible for lethal outcome of the host (204).

Residual neurological deficits, electroencephalographic changes, and psychiatric disturbances frequently persist after recovery from acute encephalitis. Pathological lesions in cases with neurological residua 12 to 67 years after recovery from acute JE were characterized by small areas of rarification (neuronal loss) surrounded by dense microglial scarring and were distributed in areas typically affected during the acute phase. In experimental animals, changes in behavior and learning ability have been documented (see Persistent and Congenital Infection, below).

Persistent and Congenital Infection

Neural tissue is a preferred site for *in vivo* persistence of latent infections with many viruses, and flaviviruses may

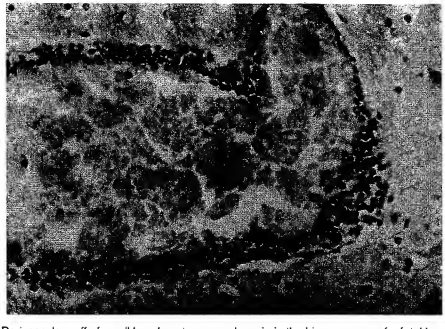


FIG. 6. Perivascular cuff of small lymphocytes around a vein in the hippocampus of a fatal human case of St. Louis encephalitis. Hematoxylin and eosin. ×400. Courtesy of M.G. Reyes.

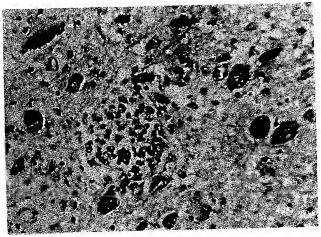


FIG. 7. Glial nodule in the substantia nigra from a fatal human case of St. Louis encephalitis. Hematoxylin and eosin. ×400. Courtesy of M.G. Reyes.

be no exception. Recent studies with alphaviruses indicate that antibody may mediate viral clearance from neurons that lack surface class I MHC determinants for recognition by cytototoxic T lymphocytes (319). The mechanism of antibody clearance appears to involve intracellular uptake of antibody and restriction of gene expression. Passive administration of antibody to mice with established flavivirus (WN virus) brain infection prevented lethal infection (402). However, the role of antibodies in viral clearance as opposed to a mechanism involving cell killing by Tc cells suggests a mechanism for persistence of viral genomes in neural cells. In the case of latent alphavirus infection of neurons, reactivation of productive virus replication is observed in the presence of waning antibody levels (320). Since abortive CNS infections with flaviviruses occur in humans and animals in nature, persistent infections may be established in neuronal tissue. In children infected with JE virus, recurrent neurologic disease has been observed (512), suggesting reactivation of latent neural infection. Chronic progressive human encephalitis and seizure disorders (Kozhevnikov's epilepsy) occurring years after infection with TBE virus has been reported (413). Yellow fever 17D virus has been recovered from the brains of monkeys >5 months after intracerebral inoculation. Hamsters and monkeys infected with tick-borne flaviviruses have developed progressive neurological degeneration with astrocytic proliferation, perivascular granulomata, and neuronal vacuolation (612,613). Chronic infection of neural or lymphoreticular tissues has also been reported in monkeys with TBE and WN viruses and in mice with Kyasanur forest disease and SLE viruses [reviewed in (379)]. In these models, some persistently infected animals either failed to develop neutralizing antibodies or reverted to seronegative, suggesting that productive viral replication occurred when immune responses were impaired or suppressed. Similarly, persistent infections with SLE, TBE, and JE viruses have been documented in hibernating bats and rodents, with reactivation following arousal, suggesting that immune suppression during hibernation may be responsible for persistence.

Virus isolates from persistently infected animals have exhibited phenotypic changes such as loss of hemagglutinin, reduced neurovirulence, and temperature sensitivity similar to that associated with persistent infection of cell cultures, indicating that persistent infections may be associated with attenuation of the virus phenotype, generation of defective interfering particles or alteration of viral antigens in order to escape immune surveillance. Inhibition of transcription by abnormal production of antisense (minus-strand) synthesis has been postulated to play a role in enterovirus and herpes virus latency, but has not been investigated in the case of flaviviruses.

In Asia, JE virus is an important cause of epizootic abortion and stillbirth in swine, and JE virus has been isolated from brain, liver, and placental tissues of these animals. Mice infected with JE virus show a high incidence of stillbirth and congenital malformations when inoculated 9 to 16 days before parturition. Mice have been shown to transmit JE virus to offspring of consecutive pregnancies, and immune suppression during pregnancy has been postulated to play a role in establishment of persistent maternal infection (356,357). Splenic T lymphocytes have been shown to harbor the virus in latently infected mice (358). Evidence for latent JE virus infection in humans is reviewed below in the section on Japanese Encephalitis)

Immune Response in Protection, Recovery, and Pathogenesis of Encephalitis

Both humoral antibody and cellular immune responses have been implicated in protection and recovery from infection. Passive transfer of monoclonal antibodies against M and E proteins (214,264,349), as well as antibodies against the NS1 nonstructural protein (502,503,505) protects mice against flavivirus encephalitis. The mechanism of protection of antibodies to NS1, which is expressed on the surface of infected cells, appears to involve Fc-dependent effector functions, but the roles of complement-mediated cytolysis and ADCC *in vivo* are uncertain (506). As mentioned above, passively transferred antibodies can abort experimental flavivirus encephalitis even when given after neuroinvasion, whereas transfer of immune spleen cells may not be effective at this stage. In severe or lethal infections characterized by high-titer viral growth and rapid accumulation of antigen in the critical target tissues, inflammatory responses may enhance lesions and accelerate death. Characterization of the inflammatory cell populations in brain tissue has shown that both NK cells and Tc cells are responsible for lysis of infected neurons and Tc cells for lysis of infected astrocytes (324,325). Infection of these target cells in the brain appears to result in an in-

terferon-induced increase in class I and II MHC antigen expression, leading to increased recognition by Tc cells. In addition to cell-mediated clearance, antibodies have been implicated in early death in mice infected with yellow fever virus, an effect attributed to complement-mediated cytolysis of infected cells (160,161).

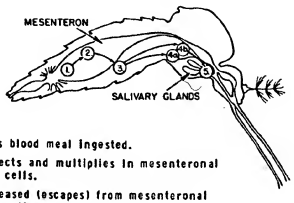
ARTHROPOD INFECTION

The dependency of certain arthropods upon blood-feeding for energy requirements and egg development provides a mechanism for salivary transmission of flaviviruses and many other infectious agents. Although mechanical transmission from host to host by simple transfer of virus on the mouthparts of mosquitoes during the process of interrupted feeding may occur occasionally, biological transmission is the rule. Biological transmission of flaviviruses by arthropods depends upon the following:

1. Ingestion of a blood meal containing virus; infection of epithelial cells lining the mesenteron (midgut)
2. Escape of virus from the midgut epithelium into the hemocele
3. Infection of the salivary gland
4. Secretion of virus in saliva during refeeding on a susceptible vertebrate host (Fig. 8)

Many flaviviruses exhibit a high degree of specificity in their ability to infect and be transmitted by individual insect or tick species (or even strains of individual species). Vector competence is under genetic control, with the susceptibility of the midgut epithelium being the primary determinant [for review, see (201,560)]. In a susceptible arthropod, a sufficient concentration of virus must be ingested to exceed the mesenteronal infection threshold. The time interval between the ingestion of an infectious blood meal and the salivary secretion of virus (extrinsic incubation period) must not exceed the life span of the arthropod. Increased temperature shortens the extrinsic incubation period, and may significantly increase the rate of virus transmission in nature. Other extrinsic factors affecting flavivirus transmission by arthropod vectors include: (a) mosquito larval crowding and nutritional deprivation, which appear to increase transmission by adult vectors; (b) rearing temperature (267); and (c) coinfection with other infectious agents such as microfilaria, which may induce lesions in the midgut epithelial barrier and thereby facilitate viral dissemination to the hemocoel (561).

Transfer of virus in mosquito saliva occurs during the process of probing host tissues with the piercing mouthparts in an attempt to cannulate a small vessel. The probing process often results in microhematoma formation, which facilitates location and cannulation of the vessel (127). Salivary enzymes play an important role in feeding, particularly apyrase, which prevents ADP-dependent platelet aggregation and coagulation (346). Salivary virus is deposited principally in the extravascular tissues of the host during probing, and saliva that is injected intravascularly is rapidly reingested by the mosquito during blood feeding (562,563). This results in virus replication at the site of inoculation and relatively slow spread of initial infection through lymphatic channels to regional lymph nodes, rather than a rapid viremic dissemination of the inoculum to the bloodstream. The delay engendered in this initial phase of virus infection may be important in the initiation of the immune response and eventual abrogation of infection. Conversely, if virus does enter the vascular space during mosquito feeding and is disseminated early in infec-



① Infectious blood meal ingested.
② Virus infects and multiplies in mesenteronal epithelial cells.
③ Virus released (escapes) from mesenteronal epithelial cells.
④ a. Virus infects salivary glands after secondary amplification in other cells/tissues.
b. Virus infects salivary glands without secondary amplification in other cells/tissues.
⑤ Virus released from salivary gland epithelial cells and is transmitted by feeding.

FIG. 8. Steps required for flavivirus infection and transmission by an arthropod. From Hardy (201), with permission.

tion, the incubation period may be abbreviated and infection accelerated. Differences in these early events in infection could partially explain variations in the course and outcome of infection in individual hosts.

For virus transmission to occur, the vector must find and successfully feed on a vertebrate host. Many factors influence the search for vertebrate hosts, including vector and host behavior and density, chemical signals emitted by the host, and environmental variables [reviewed in (127)]. By altering vertebrate host behavior, virus infection may itself influence rates of transmission, since infected animals may be lethargic and exhibit reduced defense against mosquito and tick bite. This phenomenon has been demonstrated experimentally in the case of SLE virus-infected mice (105), but its relevance under field conditions remains to be demonstrated. Interestingly, the reduced defense against mosquito attack by immature nestling birds appears to be offset by their intrinsically lower attractiveness to blood-seeking mosquitoes (510).

Maintenance of flaviviruses in nature depends on the amplification of infections during horizontal transmission, since the rate of vertical transmission to progeny is invariably low (usually <1%). Maintenance of the infection requires that the basic reproductive rate (number of new infections that arise from a single infection) be greater than one, i.e., the host infected by one arthropod must serve as the source of infection for more than one new arthropod. Since mosquitoes feed on vertebrate blood for a very brief time, infection requires that the blood meal volume contain a concentration of virus sufficient to establish midgut infection in the vector. Thus, infected vertebrate hosts involved in transmission cycles generally develop viremia titers in excess of 5 dex/mL. In contrast to mosquitoes, ticks imbibe blood over a much longer period of time. The requirement for host infection and viremia for virus amplification of tick-borne arboviruses has recently been questioned (308,410), since TBE virus is transmitted efficiently from infected ticks to uninfected ticks cofeeding on a vertebrate host in the absence of detectable viremia. Although the mechanism whereby infection of cofeeding ticks occurs has not been elucidated, it is logical to assume that the virus is blood-borne and below levels detectable by conventional techniques. Since vertebrate hosts are infected by multiple ticks in nature, virus amplification may occur without the requirement for active infection of vertebrate tissues and viremia. A component of tick saliva appears to enhance virus transmission between infected and uninfected ticks, possibly by suppressing early, nonspecific antiviral responses (NK cell activity) in the host (285,307). However, humoral immunity of previously infected vertebrate hosts would limit virus spread by the cofeeding mechanism in nature.

Vertical transmission from female arthropod to her progeny is an important mechanism for overwinter survival of certain mosquito- and tick-borne flaviviruses. Flaviviruses infect the genital tract of female mosquitoes so that the virus may enter the fully developed egg through the micropyle at the time of fertilization/oviposition (481). As opposed to true transovarial transmission (infection of the egg at the time of development in the ovary), this mechanism allows infection of mature ova during the first ovarian cycle after feeding on a viremic host. Venereal transmission of flaviviruses from male to female mosquitoes has also been demonstrated (482). The mechanism of transovarial transmission of tick-borne flaviviruses remains to be elucidated, but probably involves fundamentally different machanisms, since the adult female tick acquires infection by transstadial infection prior to egg development.

Flaviviruses generally do not damage the vector, and the pathologic changes in the midgut seen in mosquitoes infected with some alphaviruses have not been observed in the case of *Flaviviridae*. In one study, vertical flavivirus infection in *Aedes* mosquitoes appeared to induce a prolongation of development time from ova to pupa, suggesting a deleterious effect (544). However, no such effect was observed in the case of SLE virus in experimentally-inoculated *Aedes* larvae (564). These results contrast with those observed in the case of alphaviruses, which appear to induce more consistent and severe pathologic effects on mosquito larvae and pupae, possibly explaining the lower efficiency of vertical transmission of alphaviruses than of flaviviruses in their mosquito vectors.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH THE ENCEPHALITIS SYNDROME

St. Louis Encephalitis Virus

The history of SLE has been reviewed in detail by Chamberlain (75). The disease was first recognized in 1932, when an outbreak occurred in Paris, Illinois. The following year there was a large epidemic in St. Louis and Kansas City, Missouri, characterized by CNS infections of variable severity. The causative virus was isolated from human brain tissues inoculated into mice and rhesus monkeys. *Culex pipiens* mosquitoes were suspected to transmit the infection in this outbreak on epidemiological grounds, but over 20 years would pass before this species was confirmed as the principal vector in the east-central U.S. During the early 1940s the disease was recognized in the Pacific coast states, where the virus was isolated from *Culex tarsalis*. A third transmission cycle involving *Culex nigripalpus* mosquitoes was suspected during SLE outbreaks in south Florida between 1959 and 1961, and later confirmed by entomological studies. Since 1933 there have been numerous outbreaks involving the western U.S., Texas, the Ohio-Mississippi Valley, and Florida. The largest epidemic occurred in 1975, with nearly 2,000 recognized cases. Epidemic activity in the subsequent decade is reviewed in (386). In the 1980s and 1990s, SLE epidemics occurred in Colorado, California, Florida, Texas, and Arkansas (345,451,557,558).

Infectious Agent

St. Louis encephalitis virus is a member of the JE virus antigenic subgroup. Antigenic and structural properties of the virus have been described (262,554), and its nucleotide sequence has been partially elucidated (552). The organization of the genome is similar to all other flaviviruses. As expected, a higher order of sequence homology (approximately 65%) was found with members of the JE virus antigenic complex than with yellow fever or dengue viruses (approximately 40%). Cross-protection studies in experimental animals have confirmed the close relationship between SLE and other members of the JE complex, including WN and MVE viruses. However, immunization with dengue virus also confers cross-protection against SLE virus challenge in mice. During an epidemic of SLE in Florida, the incidence of encephalitis was significantly lower in persons with prior dengue infections, as determined by the presence of antibodies, than in those lacking dengue immunity (39).

St. Louis encephalitis virus produces CPE and plaques in a wide variety of cell cultures [reviewed in (262)]. High yields of virus (8 to 9 dex/mL), high grades of CPE, and efficient plaque formation are obtained in continuous cell lines of hamster (BHK-21), monkey kidney (Vero, LLC-MK_2, MA-104), and PS origin and in primary cultures of chick and duck embryo. Infectivity titers in mammalian cell cultures are comparable to those in suckling mice. Mosquito cell cultures (derived from *Aedes, Culex*, or *Toxorhynchites* spp) support growth of SLE virus, often with persistent infection and lack of CPE or with syncytium formation (448,449). Interestingly, SLE virus replicated to high titer and caused cytopathic effects in lepidopteran cells (Sf9, Spodoptera frugiperda), whereas yellow fever 17D and dengue viruses, alphaviruses, and bunyaviruses did not (607).

Infant mice and hamsters are highly susceptible to lethal infection by the intracerebral and peripheral routes. With increasing age, these hosts develop resistance to peripheral challenge but remain susceptible to intracerebral inoculation. Virus strains vary considerably in virulence for mice and may be classified as highly virulent, avirulent, or of intermediate virulence on the basis of the intraperitoneal/intracerebral LD_{50} ratio (382). Mouse virulence correlates with pathogenicity for rhesus monkeys inoculated intracerebrally and with the capacity of virus strains to induce viremia in the house sparrow (42). Chickens develop viremia without disease; newly hatched chicks have the highest titers. Young rats inoculated intracerebrally are partially susceptible, and survivors may develop cataracts (199). Guinea pigs, older rats, and rabbits develop antibody but not disease or consistent viremia. The SLE virus infects the chorioallantoic membranes of chick embryos. Horses are frequently infected in nature without known clinical consequences but are susceptible to lethal encephalitis after intracerebral inoculation of the virus.

The SLE virus has been isolated from a variety of wild vertebrate species during field studies, including many species of birds, as well as raccoons, opossums, and bats in North America and birds, rodents, nonhuman primates, and the three-toed sloth in tropical America.

Pathogenesis and Pathology

The pathogenesis in experimental animals has been described above. The neuropathology associated with human disease is reviewed by Gardner and Reyes (149). Principal lesions include neuronophagia, cellular nodules, and perivascular cuffing, most severely affecting substantia nigra, thalamus, and hypothalamus.

Clinical Features

Three clinical syndromes are described: encephalitis, aseptic meningitis, and febrile headache (Table 3). The severity of illness increases with advancing age, and persons over 60 years have the highest frequency of encephalitis. The incubation period varies between 4 and 21 days. Onset is characterized by generalized malaise, fever, chilliness, headache, drowsiness, anorexia, nausea, myalgia, and sore throat or cough, followed in 1 to 4 days by the acute or subacute appearance of meningeal and neurologic signs. Early urinary tract symptoms (frequency, urgency, dysuria) occur in nearly one-fourth of the patients (445). There is no pathognomonic profile of neurologic findings. Altered level of consciousness, abnormal reflexes, tremor, and signs of thalamic, brain-stem, and cerebellar dysfunction (nystagmus, myoclonus, ataxia) are the most prominent findings. Cranial nerve involvement may occur, particularly lower motor neuron n. VII deficit. Approximately 10% of patients have convulsions—a poor prognostic sign. Approximately 50% of patients with fatal infections die within 1 week of onset, and 80% die within 2 weeks of onset. The case-fatality rate increases with age, from 2% in young adults to over 22% in the elderly. The disease may be complicated by bronchopneumonia, bacterial septicemia, pulmonary embolism, or gastrointestinal hemorrhage. Underlying hypertensive and arteriosclerotic disease, diabetes, and chronic alcoholism predispose to severe infection and fatal outcome. Four of the 41 recognized cases of SLE during an outbreak of SLE in Houston, Texas in 1991 were in patients with human immunodeficiency virus infection, suggesting that immunosuppression caused by HIV may be a risk factor for expression of symptomatic encephalitis (416).

A number of clinical laboratory abnormalities associated with SLE infection have been described, including an elevated peripheral white blood cell count and increased serum transaminase, creatine phosphokinase, and aldolase levels. The urinalysis may show pyuria, microscopic hematuria, and proteinuria, and there may be an elevated blood

TABLE 3. *Clinical syndromes caused by infection with flaviviruses associated with CNS disease*

Encephalitis[a] (including meningoencephalitis and encephalomyelitis)
Acute febrile illness[b]
One or more signs in either of the following categories:
Altered level of consciousness (confusion, disorientation, delirium, lethargy, stupor, coma)
Objective signs of neurologic dysfunction: (convulsion, cranial nerve palsy, dysarthria, rigidity, paresis, paralysis, abnormal reflexes, tremor, etc.)
Aseptic meningitis[a]
Acute febrile illness[b]
Sign(s) of meningeal irritation (stiff neck with or without positive Kernig's or Brudzinski's sign)
No objective signs of neurologic dysfunction
Febrile headache[a]
Acute febrile illness
Headache (may also have other systemic symptoms, e.g., nausea or vomiting)
No signs of meningeal irritation or neurologic dysfunction

[a] CSF pleocytosis present in patients with encephalitis and aseptic meningitis; it may also be found in patients with the syndrome of febrile headache.
[b] Oral temperature 100°F, 37.8°C.
From Brinker, Paulson, and Monath (52), with permission.

urea nitrogen level (445). The cerebrospinal fluid (CSF) shows moderate pleocytosis (≤500 cells/mm^3), mainly lymphocytes, although polymorphonuclear cells may predominate early in the infection. The CSF protein may be elevated (usually 45 to 100 mg%, rarely as high as 500 mg%). There are a few reports of hypoglycorrhacia. Hyponatremia and hypo-osmolarity occur in up to one-third of the patients as a result of inappropriate secretion of antidiuretic hormone (590). Elevated plasma 17-hydroxycorticosteroids and loss of the normal diurnal pattern of glucocorticoid secretion indicate reaction to stress (122). A disproportionately high cerebral perfusion in relation to metabolic demands has been found, indicating a disturbance in cerebral autoregulation of blood flow, which was unrelated to changes in sensorium of acutely ill patients. Radionuclide brain scans and computed tomography have been normal. The electroencephalogram shows diffuse generalized slowing and amorphous generalized delta wave activity (52).

A period of prolonged convalescence occurs in 30% to 50% of cases, characterized by asthenia, irritability, tremulousness, sleeplessness, depression, memory loss, and headaches, lasting up to 3 years. Approximately 20% of these patients have symptoms persisting for longer periods, including gait and speech disturbances, sensorimotor impairment, psychoneurotic complaints, and tremors (136). Old age and severity of acute illness appear to predispose to these sequelae.

Jaundice was reported in two cases of SLE occurring in Brazil (568). Hepatitis has also been a feature of WN virus infections in the tropics, raising the possibility that the clinical spectrum of infection with members of the JE virus complex may be broader than currently appreciated.

Diagnosis

The patient's age, season of the year, place of residence and exposure, and information about the occurrence and serodiagnosis of similar cases in the community are of paramount importance in the differential diagnosis. In the individual case, it is essential to rule out treatable bacterial, mycobacterial, spirochetal, and fungal infections as well as herpes encephalitis. Because SLE often strikes the elderly, it has occasionally been misdiagnosed as stroke.

Virus isolations from serum or CSF are very unusual, and testing is not profitable. In over one-half of fatal cases, virus may be recovered by intracerebral inoculation of suckling mice with suspensions of brain tissues; occasional isolates have been made from liver, spleen, lung, and kidney (67). St. Louis encephalitis viral antigen has been demonstrated by careful immunofluorescence examination of brain frozen sections (458). Flavivirus-like particles have been found by electron microscopy, and SLE antigen has been found by immunofluorescence in urine sediment (335). Polymerase chain reaction assays applied to blood, CSF, or tissue may provide a sensitive approach to early diagnosis in the future.

Specific diagnosis usually relies on serological tests on appropriately timed acute and convalescent samples. The HI test detects mainly group-reactive antigens and is a useful screening procedure. Antibody titers increase rapidly during the first week after onset. In primary infection, titers to SLE antigen are usually higher than to heterologous antigens. In some areas of the southern U.S., cross-reactions due to immunity to dengue virus confuse the diagnosis in older individuals. Complement fixing antibodies appear during the second week and peak at 3 to 4 weeks after onset. Because CF antibody titers then fall off to low levels by 9 to 12 months, a diagnosis can often be made by demonstrating a fourfold or greater fall in titer between early and late convalescent sera. The presence of CF antibody in a single serum sample is presumptive evidence of a recent infection. However, 20% of patients with confirmed SLE virus infections fail to develop detectable CF antibodies (67). Cross-reactions in the CF test are less than by HI. The neutralization test is most specific. Antibodies appear during the first week and persist for many years, usually for a lifetime.

Local production of IgM antibodies in the CNS provides a potential means of rapid and early diagnosis. Demonstration of IgM antibodies in CSF by enzyme-linked immunosorbent assay (ELISA) appears also applicable to the diagnosis of SLE as early as 3 to 5 days after onset. The IgM-capture ELISA has replaced classic serologic methods in many laboratories. IgM antibodies in serum appear within the first 4 days after onset, peak at 7 to 14 days, and decline thereafter, generally reaching extinction by 60 days

(385). Presence of IgM antibody in a single serum is presumptive evidence of recent infection. However, because IgM antibody may persist for up to a year in up to one-fourth of patients, demonstration of a decrease in antibody titer between paired sera is preferable. IgA antibodies parallel those in the IgM class (558).

Treatment

Neither antiviral chemotherapeutic agents nor interferon have been evaluated for therapy of SLE. Treatment is supportive and consists of good general management and nursing care, especially in the semicomatose and comatose patient.

Hyponatremia secondary to inappropriate antidiuretic hormone (ADH) secretion is managed with water restriction. Marked, progressive elevations in intracranial pressure have not been documented in SLE, but this possibility should be considered in severely ill patients with deepening coma and loss of brain-stem reflexes. Anticonvulsant therapy may be required.

Epidemiology

Morbidity and Mortality

The epidemiology of SLE is reviewed in (373,386,451, 555). Since the inception of nationwide surveillance in 1955, nearly 5,000 cases of SLE have been officially reported in the U.S. (Fig. 9). Major outbreaks, which occur every 5 to 15 years have involved up to 1,815 officially notified cases. Endemic transmission occurs during interepidemic intervals, with small numbers of notified cases (<50 per year). Attack rates in localities affected by epidemics have ranged from 1 to 800 per 100,000 population. The overall case-fatality ratio is approximately 7%, but it is significantly influenced by age. The ratio of inapparent

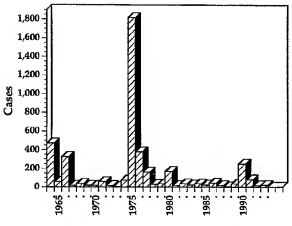


FIG. 9. Annual incidence of St. Louis encephalitis in the U.S., 1964–1993. Data from Division of Vector-Borne Infectious Diseases, Centers for Disease Control.

to apparent SLE infection varies with age, from 806:1 in children to 85:1 in the elderly.

The disease appears in July, with the peak incidence in August and September, but outbreaks may occur later in the year at southern latitudes. St. Louis encephalitis predominantly affects the Ohio-Mississippi Valley, eastern Texas, Florida, Kansas, Colorado, and California (Fig. 10). The primary transmission cycle involves wild passeriform and columbiform birds and the *Culex* spp. mosquitoes (Fig. 11). Mammals do not participate in the primary transmission cycle, but occasional isolates have been made from carnivores, and bats have been suggested to play a role in overwinter maintenance. The vectors responsible for virus amplification between birds and transmission from birds to humans vary regionally. In the Ohio-Mississippi basin and eastern Texas, the distribution of cases is urban-suburban, corresponding to high densities of the principal vectors, *Culex pipiens*, and *Culex quinquefasciatus*, which

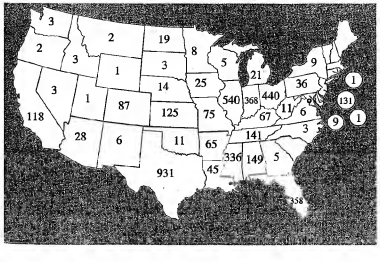


FIG. 10. Distribution of St. Louis encephalitis cases in the U.S., 1964–1993. Data from Division of Vector-Borne Infectious Diseases, Centers for Disease Control.

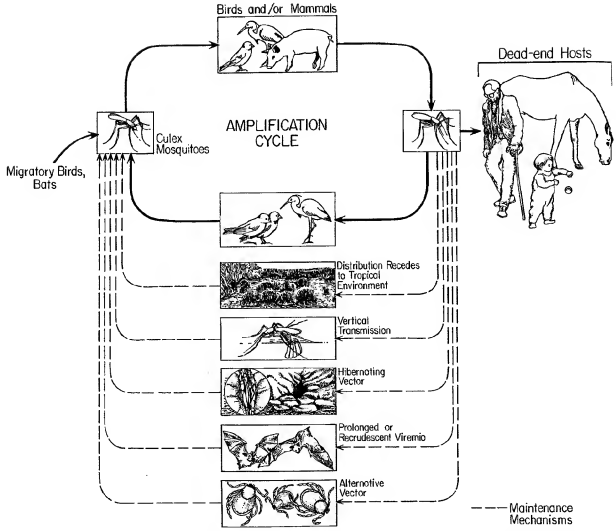


FIG. 11. Generalized transmission cycle of mosquito-borne flaviviruses causing encephalitis, showing summertime amplification and presumed overwintering mechanisms. Humans are dead-end hosts and do not participate in perpetuation of virus transmission. Vector species vary, but culicine mosquitoes (principally *Culex* spp.) are responsible for amplification cycles. Wild birds are the most important viremic hosts for most viruses, but in the case of Japanese encephalitis, pigs play an important role. The pattern shown applies to St. Louis, Japanese, Murray Valley and Rocio encephalitis viruses, West Nile, Kunjin, Usutu, and possibly other flaviviruses (see Table 1).

breed in polluted water (especially where poor sanitation exists). In Florida the tropical mosquito *Culex nigripalpus* is the epidemic vector. In the western states the principal vector, *Culex tarsalis*, breeds in irrigated or flooded dryland areas; its wide distribution leads to frequent human exposures in rural areas. *Culex tarsalis* is also the vector of western equine encephalitis virus, and transmission of this virus and of SLE are often concurrent (451). St. Louis encephalitis generally occurs as a rare, sporadic disease in many areas of tropical America (527).

In the eastern and central U.S. the incidence of disease and the case-fatality rate are significantly higher in individuals over 55 years than in younger persons. Although previously obscured by a high background of naturally acquired immunity in endemic areas of the western U.S., the greater susceptibility of the elderly has become evident in the West. Altered behavioral patterns of the human population (use of air-conditioning and television) may be responsible for decreased exposure to mosquitoes, as well as a decline in infection and immunity, in these endemic areas (145).

In rural areas of the western states, where *Culex tarsalis* is the principal vector, cases in males outnumber those in females nearly 2:1 because of the greater opportunity of

exposure of males working outdoors. A predominance of cases in females has been found in the central and eastern states, where exposure to the household mosquito vectors (*Culex pipiens, Culex quinquefasciatus*) is responsible for infection. *Culex restuans* may be involved in early transmission during the cool spring months, preceding amplification by *Culex pipiens/quinquefasciatus*. In most *Culex pipiens*-borne outbreaks in the southern U.S., the incidence has been highest in black populations inhabiting lower socioeconomic areas of affected cities where environmental conditions favor breeding of the vector. Risk factors for infection associated with low socioeconomic status include living in homes near open storm sewers and without screens and air conditioning, and sitting outside in the evening (345,373). In Florida, however, the black population was relatively spared during epidemics between 1959 and 1962; the higher prevalence of antibodies to dengue virus in the elderly black population may have provided a degree of protection (39).

Although historically a disease of rural agricultural communities in the western U.S., SLE appeared in epidemic form in Los Angeles in 1984 (451). Subsequent investigations showed that virus transmission occurred principally in residential habitats, parkland, and vegetated undeveloped areas, with *Culex tarsalis* as the principal vector (456).

Immunity

Surveys in several urban areas in the eastern U.S. have shown a 6% incidence of SLE virus infection during epidemics (229). A survey of urban and rural Indiana residents showed an overall seroprevalence of 3.6% and an estimated annual infection rate of 0.32% (171). A serological survey in Los Angeles County during the outbreak in 1984 indicated evidence of infection in 1.6% of the human population.

Origin and Spread of Epidemics

Evidence indicates that SLE virus is maintained in local winter reservoirs in North America, but the possibility of annual reintroduction from warm regions of year-round transmission by migratory birds or bats cannot be excluded. The virus has been isolated from hibernating adult female *Culex pipiens* (17), and there is annual recrudescence of virus activity throughout much of North America. Vertical transmission of virus has been experimentally shown in *Culex pipiens, Culex quinquefasciatus, Culex restuans*, and other species. The virus can also be transmitted venereally in *Culex*, providing a potential mechanism for virus spread from vertically infected males to female mosquitoes (514). St. Louis encephalitis virus has been isolated from the gizzard of a cowbird 38 days after experimental infection (76) and from the blood of bats for prolonged periods (537). Transplacental passage of virus in bats has also been demonstrated. Despite these observations, the mechanism(s) whereby SLE virus is maintained locally over the winter will require further elucidation.

TABLE 4. *Some factors that affect rates of flavivirus transmission in nature*

Virus
Strain differences in infectiousness for vectors and hosts
Vectors
Susceptibility to infection and efficiency of transmission ("vector competence")
Vector population density, population dynamics, and age structure
Longevity
Preference for biting host species
Distribution and dispersal (flight range)
Reservoir–hosts
Susceptibility to develop effective viremia
Host population density, population dynamics, and age structure
Active and passive immunity
Attractiveness to vectors
Distribution and dispersal
Clinical hosts (e.g., humans)
Population density
Acquired immunity
Exposure to vector bites

Modified from Reeves (451).

Amplified transmission of the virus begins in the springtime and early summer, with the reemergence and breeding of vector mosquitoes. If conditions are favorable, a rapid, cumulative, and progressive increase in the transmission cycle follows, involving the epidemic vectors. Wild passerine birds serve as the main viremic hosts in these cycles of transmission. A comprehensive review of the avian species involved is given by McLean and Bowen (367). If the rate of virus transmission between birds and mosquitoes is sufficiently high, humans and other mammals (horses, dogs, etc.) may be tangentially infected but do not serve as viremic hosts. Many factors influence the rate of spread of virus (Table 4) and determine the course and extent of epidemics.

Molecular Epidemiology

St. Louis encephalitis virus strains exhibit extensive genomic variability (553). Strains can be classified by their geographic origin; moreover, within a given area, both genetic drift and introduction of other geographic types have been demonstrated over time. Genome analysis thus provides a possible means of determining the origin and source of outbreaks. The presence of three unique genotypes defined by RNAase T_1 oligonucleotide fingerprinting—(I) Florida (associated with the *Culex nigripalpus* cycle); (II) the Ohio-Mississippi River basin and eastern Texas (*Culex pipiens/ quinquefasciatus*); and (III) the western United

States (*Culex tarsalis*)—suggests that the virus is maintained in each area in local reservoir hosts or vectors. Although relatively few strains from tropical America have been studied, they appear to represent distinct genotypes, again suggesting that the annual recrudescence of SLE virus transmission in North America is not dependent upon reintroduction by migratory birds or bats. The segregation of North American virus genotypes by mosquito vector has evolutionary implications, and the selective forces have been partially elucidated (42,382,553). Virus genotype III is transmitted principally by *Culex tarsalis*, despite the sympatric distribution of ample populations of of *Culex quinquefasciatus* in the western U.S. This virus genotype is, however, less virulent than genotype II for a variety of hosts, including avian species, which develop relatively low viremias. Since *Culex tarsalis* is highly susceptible to oral infection, whereas *Culex quinquefasciatus* exhibits lower susceptibility, the former species is capable of amplified transmission of genotype III, whereas the latter is not. In the central U.S., virus genotype II elicits high viremias in birds, sufficient to readily infect the more refractory *Culex pipiens* and *Culex quinquefasciatus*. Since *Culex tarsalis* is not prevalent in this region, selective pressure for virulence is maintained on the virus genotype by the restricted vector competence of *Culex pipiens* and *Culex quinquefasciatus*.

Prevention and Control

At the present time, no vaccine against SLE is available (573). Reduction of vector populations remains the most widely used method for prevention and control of SLE epidemics. Surveillance programs focus on early detection of increased levels of virus activity by testing avian sera for antibodies or mosquitoes for virus or viral genome by PCR. Sentinel flocks of chickens followed at frequent intervals for serologic conversions provide a sensitive indicator of SLE virus activity and have accurately predicted impending human epidemics.

Japanese Encephalitis Virus

A disease resembling JE was recognized in horses and humans as early as 1871. A severe epidemic occurred in Japan in 1924, and a filterable agent was extracted from human brain and passed to rabbits, but the agent was not identified or characterized. In 1934, Hayashi reproduced the disease in intracerebrally inoculated monkeys. In 1935, the agent was recovered from the brain of a human in Tokyo and was virologically and serologically established as the prototype (Nakayama) strain. The virus was first recovered from brain tissue of a sick horse in 1937. Mosquito transmission was suspected during the early 1930s; in 1938, Mitamura et al. reported isolation from *Culex tritaeniorhynchus*. Classic studies in Japan by Scherer et al. (56,497) established that pigs and birds were the principal viremic hosts and that *Culex tritaeniorhynchus* was responsible for transmission between these vertebrates and from them to humans.

Epidemics of JE recur in temperate areas of Asia and in the northern part of tropical Southeast Asia. In terms of morbidity and mortality, this disease is by far the most important of the arbovirus encephalitides [for reviews, see (556,565)].

Infectious Agent

Japanese encephalitis virus is the prototype of the JE antigenic complex. Cross-reactions in neutralization test and cross-protection in animals has been demonstrated between JE and other flaviviruses, particularly members of the JE complex. The complete nucleotide sequence of the JE viral genome has been determined (361,540), and JE viral antigens have been expressed in recombinant systems (144,277,350,529). A full-length infectious clone was constructed by *in vitro* ligation of cDNA fragments (541). Antigenic variation among JE strains has been shown by antibody-absorption HI, agar gel diffusion, CF, kinetic neutralization, antibody-absorption neutralization (411), and monoclonal antibody analysis (275). At least two immunotypes have been repeatedly distinguished: Nakayama (representing the prototype strain isolated from human brain in Japan in 1935) and JaGAr 01 (from *Culex* mosquitoes, Japan, 1959). Virus strains isolated in 1969 to 1970 were immunologically placed between these types. To further complicate matters, antigenic differences have been shown between substrains of Nakayama virus. The recognition of antigenic strain variation has led to altered strategies for vaccine preparation (see Prevention and Control, below). Geographic variation between JE virus strains has been demonstrated by examination of RNA fingerprints and nucleotide sequencing (see Molecular Approaches, below).

Considerable variation exists in neurovirulence and peripheral pathogenicity for mice among JE virus strains (240,241). Virus strains recovered from brain tissues of fatal human cases in Thailand differed by RNA fingerprint analysis from contemporary strains from pigs and mosquitoes (64), suggesting that the human isolates represent distinct neuroinvasive phenotypes. Virus strains from Malaysia and Indonesia, where JE occurs as a endemic infection with sporadic cases, are dinstinguishable by nucleotide analysis (83) from strains elsewhere in Asia, where the disease causes epidemic disease, suggesting that the Indonesian strains may have reduced virulence for humans.

The virus replicates in a wide variety of primary and continuous cell cultures of hamster, porcine, chicken, monkey, and mosquito origin. Vero and LLC-MK$_2$ cells are useful for plaque assays (531). Infant mice are highly susceptible to lethal infection by all routes of inoculation.

Weanling mice succumb to intracerebral virus inoculation, but there is virus strain variation in pathogenicity by the intraperitoneal route. Hamsters and monkeys die after intracerebral or intranasal inoculation but develop asymptomatic viremia after peripheral infection. Rabbits and guinea pigs have asymptomatic infections by all routes of inoculation. The virus is pathogenic for embryonated chicken eggs. It produces disease in horses and swine (see below), whereas cattle are not susceptible.

Pathogenesis and Pathology

Sites of replication and dissemination of virus in the mouse have been described by Huang and Wong (241). During the acute stage, congestion, edema, and small hemorrhages are found in the brain. Microscopic lesions include neuronal degeneration and necrosis, neuronophagia, microglial proliferation forming glial nodules, and perivascular inflammation. These changes occur in gray matter and predominantly affect diencephalic, mesencephalic, and brain-stem structures. Destruction of cerebellar Purkinje cells may be prominent. A variety of pathological changes in extraneural tissues have also been noted, including hyperplasia of germinal centers of lymph nodes, enlargement of malpighian bodies in spleen, interstitial myocarditis, swelling and hyaline changes in hepatic Kupffer cells, pulmonary interalveolitis, and focal hemorrhages in the kidneys.

In one study of fatal human cases, JE viral antigen was localized to neurons, with no evidence for glial cell infection (260). The highest concentration of infected neurons was in thalamus and brain stem. Among inflammatory cells recruited into perivascular infiltrates, Th cells predominated, but a minority were T-suppressor/cytotoxic lymphocytes. Macrophages predominated among cells recruited into the brain parenchyma.

Dual human infections with JE and herpes simplex viruses have been described. In analogous experiments in mice, JE viral antigen was localized in herpesvirus-infected areas of the brain, suggesting that JE virus gained access to the CNS at sites of blood-brain barrier disruption caused by herpesvirus (210). A similar role has been implicated for *Toxocara canis* and *Trichinella* in experimental dual infections (98,424) and for *Taeniasis solium* (neurocysticercosis) in humans (102,511).

Transplacental infection in swine results in fetal encephalitis, abortion, and stillbirth. The virus also produces hypospermia and aspermia in boars (186). Histopathological changes include epididymitis, spermatogenic arrest, and inflammation of the tunica testis. Pregnant mice inoculated intraperitoneally also transmit JE virus to the fetus, with subsequent abortion (356,357). A curious feature of this model is that infected mothers, when mated again after 6 months, transmitted virus to the second litter. Latent infections of pregnant mice could be reactivated by cyclophosphamide or subsequent pregnancy. In other studies, latent infections were detected by cocultivation of mouse spleen cells and virus located principally to Lyt 1 cells (358).

There is also evidence for congenital and persistent infections in humans. In a series of nine pregnant women infected during an epidemic, four women infected during the first and second trimesters aborted and virus was isolated from fetuses, whereas none of the five women infected in the third trimester miscarried or had abnormal babies (556). No other reports of human congenital infection are extant, and the frequency of this complication is unknown. Most women of child-bearing age in endemic areas of Asia are naturally or artificially immunized during childhood and are therefore not at risk. Evidence for latent infections of humans has been reported. By use of cocultivation techniques, JE virus was recovered from peripheral blood mononuclear cells of several children who developed recurrent disease as well as asymptomatic children 9 months after an acute JE infection (512). Virus has been recovered from cerebrospinal fluid samples as late as 117 days after onset of clinical symptoms (450). The frequency with which latent or persistent extraneural or neurological infection occurs, whether such infections are associated with cytopathology or clinically relevant syndromes, and how the immune response modulates such infections remain to be elucidated.

Clinical Features

The incubation period is 6 to 16 days. As in SLE, illness may be manifested by a febrile headache syndrome, aseptic meningitis, or encephalitis (116,151). In the full-blown encephalitic form, onset is rapid, beginning with a 2- to 4-day prodromal phase of headache, fever, chills, anorexia, nausea and vomiting, dizziness, and drowsiness. In children, abdominal pain and diarrhea may be prominent. These symptoms are followed by the appearance of nuchal rigidity, photophobia, altered states of consciousness, hyperexcitability, and varying objective neurological signs, including dull, masklike facies, muscular rigidity, cranial nerve palsies, tremulous eye movements, coarse tremors of the extremities, involuntary movements, generalized and localized paresis, incoordination, and pathologic reflexes. Sensory deficits are rare. Paralysis of the upper extremities is more common than paralysis of the legs. Spinal cord involvement (lower motor neuron deficits) may occur, and a bulboparetic syndrome has been described. Convulsions are frequent in children but occur in less than 10% of adult patients. Severe hyperthermia may require specific countermeasures. Death occurs on the fifth to ninth day or during a more protracted course with cardiopulmonary complications. A poor prognosis is associated with respiratory dysfunction, positive Babinski's sign, frequent or prolonged seizures, prolonged fever, albuminuria, and evidence for high levels of virus replication in the brain, including in-

fectious virus in CSF, high levels of α-interferon in CSF, and low levels of IgM and IgM antibodies in serum and CSF (59).

The peripheral white blood cell (WBC) count is mildly elevated. Urinary tract symptoms are common during the acute phase of illness and may be accompanied by sterile pyuria and microscopic hematuria and albuminuria. The CSF pressure may be elevated; microscopic examination shows 10 to 500 (rarely up to 1,000) WBCs, with neutrophils predominating early followed by a typical lymphocyte response. Cell counts fall gradually over 8 to 9 weeks. The CSF protein concentration is mildly elevated (50 to 100 mg%). The electroencephalogram (EEG) is abnormal, with decreased electrical activity, slowing, and dysrhythmia. Brain echo and cranial tomography performed during the acute phase have shown characteristic thalamic and basal ganglia abnormalities correlating with poor prognosis.

The case-fatality rate is 5% to 40%. Higher rates during epidemics (up to 70%) are reported but reflect poor medical care and recognition of only the most severe cases. Case-fatality rates in affected American servicemen varied between 2% and 11%. Children and the elderly are at highest risk of fatal infection. Neuropsychiatric sequelae occur in 45% to 70% of survivors and are particularly severe in children (288). Sequelae include parkinsonism, convulsive disorders, paralysis, mental retardation, and psychiatric disorders. The social prognosis of survivors is generally poor. Sequelae are more frequent in patients whose acute disease is severe, prolonged, and associated with coma and localizing neurological signs. Magnetic resonance imaging in patients with sequelae has revealed abnormal intensity areas in thalamus, globus pallidus, hippocampal areas, and substantia nigra providing anatomical correlates of the emotional lability, memory impairment, and parkinsonism.

Diagnosis

Definitive diagnosis is similar to that described for SLE. In fatal cases, virus isolation and demonstration of viral antigen by fluorescent antibody in brain tissue is feasible. Rapid diagnosis may be achieved by immunofluorescent staining of antigen in mononuclear cells recovered from CSF (359). Virus isolation from blood is infrequent. However, virus may be isolated from CSF in up to one-third of patients during the acute phase, and isolation is correlated with a poor prognosis. Serologic diagnosis depends on the demonstration of a fourfold or greater rise (or fall) in appropriately timed serum specimens. The HI, CF, and neutralization tests are applicable. Cross-reactions with heterologous viruses, particularly WN, may complicate the serodiagnosis in tropical areas of Asia. Serum IgM antibodies appear early, usually disappear by 3 to 6 months after onset, and are usually serologically specific (60). The IgM-capture ELISA is especially well suited for diagnosis by detection of locally synthesized antibody in the CSF (60,63), thus separating patients with virus infection of the CNS from those with other etiologies of encephalitis but serologic evidence for systemic (extraneural) JE infection. Intrathecal antibody synthesis can also be estimated by use of an IgG index or CSF IgG/CSF albumin ratio. The kinetics of the systemic and local CNS antibody response have prognostic importance; patients who survive have earlier and more vigorous responses. IgM antibody synthesis may persist in CSF for weeks after recovery, indicating persistent antigenic stimulation (450). Monoclonal epitope-blocking immunoassays may be used to identify JE-immunes among persons with cross-reactive flavivirus antibodies (61).

Treatment

There is no specific treatment. Good supportive care (as discussed for SLE) is essential. Vigilance should be maintained for severe hyperthermia and convulsions, and specific countermeasures should be applied. Cerebral edema has been postulated to be an important factor in pathogenesis. However, a controlled trial of high-dose corticosteroid therapy failed to show improvement in clinical outcome, mortality, or sequelae (235). A technical guide to the clinical diagnosis and management of patients has been prepared by the World Health Organization (600).

Epidemiology

Morbidity and Mortality

Japanese encephalitis virus is widely distributed in Asia, including Japan, China, Taiwan, Korea, Philippines, far-eastern USSR, all of Southeast Asia, and India (Fig. 12). Approximately 35,000 cases and 10,000 deaths are recognized annually throughout Asia, but the disease is greatly underreported. The incidence in Japan, South Korea, and Taiwan has declined dramatically in the last 20 years. Large numbers of cases (>10,000/year) occur in China, but the annual incidence is also declining. In contrast, epidemic activity in northern and central India, Nepal, and the northern part of Southeast Asia has increased since the early 1970s. In one study of 740 consecutive hospital admissions with encephaopathy in India, 23% were due to JE virus, and 37% of these patients died (287). In northern Thailand and in Vietnam outbreaks occur with attack rates of up to 10 to 20 per 100,000, an incidence similar to that of poliomyelitis in the U.S. before the advent of vaccines (234). The increase in JE virus transmission in these areas is linked to deforestation, agricultural development, and irrigation schemes for rice cultivation, leading to high-density breeding of *Culex tritaeniorhynchus* (426). A recent episode in the island of Saipan illustrated the dynamics of JE infection after introduction of the virus into a susceptible area

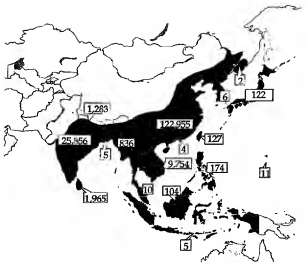


FIG. 12. Distribution of Japanese encephalitis (*JE*) virus (*shaded area*) and numbers of reported cases of human encephalitis due to JE virus, 1986–1990. From (13), with permission.

outside of the endemic zone (423). Virus introduction was followed by intense amplification, universal infection of the pig population, infection of 4.2% of the human population, and a disease attack rate of 25 per 100,000.

Children are primarily affected by the disease; attack rates in the 3- to 15-year age group are 5 to 10 times higher than in older individuals because of high background immunity in the older age groups. Epidemics in nonendemic regions have affected all age groups, but a bimodal age distribution (young children and the elderly) has appeared, indicating an increased risk in the elderly as seen in SLE.

In endemic areas, nearly all persons have sustained infection by young adulthood. The ratio of inapparent to apparent infections is 200:1 to 300:1 (28). Among factors which influence this ratio are age, differing virus strain virulence, and cross-protective immunity to other flaviviruses, especially dengue.

In tropical areas there is an endemic pattern of infection, with occurrence of sporadic cases throughout the year. In temperate zones and in the northern part of the tropical zone, outbreaks have a marked seasonal incidence during the rainy (monsoon) season (July to September). Precipitation and temperature are important determinants of vector density and rate of virus transmission. The incidence is highest in rural agricultural areas. An excess of cases has been noted in males in many outbreaks, presumably because of increased exposure in areas of rice cultivation.

The reasons for the absence of epidemics in tropical countries are poorly understood. In southern Thailand, intense JE virus transmission to pigs has been demonstrated in the absence of epidemic human disease (64). The RNA genome of virus isolates from this area differed significantly from northern Thai epidemic strains, suggesting that a difference in virulence may be responsible. Other factors to be considered include vector-host relationships, vector abundance, vector competence, and cross-protecting heterologous immunity to dengue.

Transmission Cycle

Birds and pigs are effective viremic amplifying hosts, serving as the source for infection of mosquito vectors (Fig. 11) (497). Cattle are frequently infected but have low viremias and do not perpetuate virus transmission (244). In temperate regions, virus transmission is detectable in July in mosquitoes, pigs, and birds, principally ardeid species (egrets, black-crowned night herons) and possibly ducks. Human infections occur several weeks later. The main epidemic vectors are *Culex* species, of which *Culex tritaeniorhynchus* is the most important. It breeds in irrigated rice fields, shallow ditches, and pools, with peak population densities during the monsoon season. Infection rates in *Culex tritaeniorhynchus* may exceed 1% during periods of peak transmission. Other species implicated in transmission include *Culex vishnui* (India), *Culex gelidus* and *Culex fuscocephala* (India, Malaysia, Thailand), *Culex annulus* (Taiwan), and *Culex annulirostris* (Guam). Isolations have been made from many other species of *Culex*, *Anopheles*, and *Aedes*.

The overwinter maintenance of JE in temperate areas has not been fully elucidated. Vertical transmission of JE virus by *Culex* and *Aedes* species has been demonstrated in the laboratory and by field studies (419,478,484). The virus is also sexually transmitted from male to female mosquitoes. Other possible mechanisms include the following: survival in hibernating adult female *Culex tritaeniorhynchus*; maintenance in ticks, mites, or other alternate vectors; persistent infections in vertebrates; and reintroduction by migrating birds. Japanese encephalitis virus and antibodies have been found in bats in Japan. Experimentally infected bats held in simulated hibernation develop persistent infections and, upon warming, have viremias sufficient to infect mosquitoes. Japanese encephalitis virus and antibodies have been found in reptiles (513), but their role in the ecology of JE remains unknown. Certainly, the demonstration that latent and recrudescent infections occur in experimentally infected mice as well as in humans lends credence to the view that wild or domestic vertebrate hosts could play a reservoir role in overwinter maintenance. In tropical regions, year-round transmission of the virus between mosquitoes, birds, and pigs probably occurs (173).

Japanese Encephalitis in Travelers.

Cases of JE have occurred among travelers to Asia. Between 1978 and 1992, 24 such cases were reported (13). Although the risk of overt disease among travelers is low

(estimated to be 1:5000 to 1:20,000/week of travel, varying with location of travel, season, and rural exposure), the severity of the disease has encouraged use of vaccine (see Control, below). Among JE cases with known outcome among travelers, 40% died and 36% had residual diabilities.

Molecular Epidemiology

Japanese encephalitis virus diversity has been determined by primer-extension sequencing of a region in the C/prM gene (83,242). These analyses demonstrated four geographically distinct genotypes of JE virus in Asia differing by >12% at the nucleotide level. Genotype I consists of virus strains from a broad region encompassing northern Asia (Japan, China, Taiwan), Vietnam, Nepal, India, and Sri Lanka. Genotype II consists of strains from northern Thailand and Cambodia. A third genotype contains strains from Indonesia (Sarawak, Java), Malaysia, and southern Thailand. A fourth comprises isolates from eastern Indonesia (Java, Bali, and Flores). It is of considerable interest that an epidemic pattern of disease expression prevails in areas where genotypes I and II circulate, but in an endemic pattern in areas represented by genotypes III and IV. Whether this reflects a difference in virus virulence or in epidemiological/ecological factors remains uncertain. The very large and continuous geographic distribution of genotype I (from Japan to Sri Lanka) suggests the possibility of gene flow, but the virus distribution does not appear to fit patterns of bird migration.

Control

Vaccination

Vaccines have been used to prevent (a) encephalitis in horses and humans and (b) abortion and stillbirth in swine. Vaccination of horses with formalin-inactivated vaccines was the first successful application and afforded significant protection during an epizootic in Japan during 1948 to 1949. Since 1972, live attenuated vaccines have been licensed in Japan for use in pigs (556,603). Although immunization of pigs is a theoretical means of interrupting transmission and amplification of JE virus and thereby of preventing human infections, difficulties arise in practice. In many parts of Asia, pigs are only semidomesticated, and wide-scale immunization is difficult. In areas such as Japan, where swine husbandry is highly developed, pigs are born after the epidemic period, have interfering maternal antibody for 4 months, and are killed the following summer, leaving a narrow interval for vaccination.

Formalin-inactivated vaccines for use in humans are prepared from infected adult mouse brains or infected primary hamster kidney cell cultures in Japan and China, respectively [for review, see (556)]. The mouse brain vaccine produced by the Research Foundation for Microbial Diseases (Biken), Osaka, Japan, is purified by protamine sulfate precipitation and ultracentrifugation and has been in wide use since the 1960s. A controlled trial of Biken vaccine in Thailand showed an efficacy of 91% (234). Mass vaccination campaigns have been carried out in Japan, Taiwan, and China, with children as the target population. Primary immunization requires at least two doses of 1.0 mL (0.5 mL in infants <3 years) at a 7- to 14-day interval. Booster vaccinations are given during the first year after primary immunization and then at 3- to 4-year intervals.

Because of the antigenic diversity of JE virus strains, efforts were made by Biken to improve upon the original mouse brain vaccine produced from the 1935 Nakayama virus by incorporating a more recent isolate, Beijing-1 virus, representing a distinct (JaGAr-01) antigenic variant. Immunization of mice with Beijing-1 vaccine raised antibodies that were cross-reactive at higher titer with JE strains of different geographic origin. The Beijing-1 strain also replicates to higher titer in mouse brain. It is more potent and may be administered at a dose one-half that of Nakayama or bivalent vaccine. A monovalent Beijing-1 vaccine is currently produced for use in Japan, and it may ultimately replace the bivalent product for export. A comparison of Nakayama and Beijing vaccines in nonimmune Thai schoolchildren showed ≥94% seroconversion against the homologous virus strain (476). At this time, there is no evidence to suggest that antigenic variation of vaccine or wild virus has a significant effect on vaccine efficacy.

Since 1981 when JE was proved to be a threat to travelers, there has been an international interest in vaccines. The Biken vaccine is licensed by FDA for use in the U.S. (marketed by Connaught Laboratories) and in Europe (Pasteur-Merieux). Studies in adults have shown that three (rather than two) inoculations are required to achieve protective immunity (neutralizing antibody titers >10) in >90% of the vaccinees. Booster doses are recommended after 12 months. Side effects are generally inconsequential, consisting of local tenderness or mild systemic symptoms in 10% to 30%. Although produced from brain tissue of mice, the purified vaccine contains <2 ng/mL myelin basic protein, an amount far below that capable of eliciting allergic encephalomyelitis in the sensitive guinea pig model. Only very rare cases of postvaccinal encephalitis have been reported (414), and in such cases, a clear etiological role for JE vaccine has been difficult to establish. Surveillance for postvaccinal neurological events in Japan has not demonstrated an association between JE and neurological complications.

Use of JE vaccine in adult travelers during the last decade has resulted in a number of cases of allergic reactions, including urticaria, angioedema, bronchospasm, and erythema nodosum and multiforme (13). These reactions occurred acutely or after an interval of up to 7 days. The delay in onset appeared to lengthen when reactions followed the second or third vaccine inoculation. The incidence of these allergic phenomena has varied in different reports from 2

per 1,000 to as high as 1%. Allergic reactions in Western adult vaccinees appear to have increased in recent years, a phenomenon that was not explained by changes in vaccine manufacture or by use of specific vaccine lots.

Two other JE vaccines are currently in use in China: formalin-inactivated vaccine produced from primary hamster kidney (PHK) cells, and a live attenuated vaccine designated *SA14-14-2*, also prepared in PHK cell culture. Both vaccines are extensively used in China. Immunogenicity of the live vaccine in children exceeds 95%, but antibody titers appear to wane rapidly, and a booster dose is given at 12 months.

Efforts are underway to produce genetically-engineered JE vaccines. Recombinant vaccinia (including the NYVAC vector, a highly attenuated strain of vaccinia suitable as a vaccine) encoding prM and E (or preM, E, and NS1) of JE virus elicited neutralizing antibodies and conferred a high level of protection against challenge of mice and pigs (278,279). Recombinant vaccinia vaccines expressing only the NS1 protein showed low levels protection (277). Infection of mammalian cell cultures with recombinant vaccinia virus expressing the prM and E genes of JE virus results in the production of extracellular 20-nm subviral particles. These particles, composed of the prM/M and E viral proteins in a lipid bilayer, are free from nucleic acid, are easily purified, and are highly immunogenic, providing a practical approach to vaccine development (352). A full-length infectious clone of JE virus has been developed, allowing the introduction of attenuating mutations (541), and a potential means of constructing new live vaccines. Novel carrier/delivery systems for subunit or purified virion JE vaccines have been investigated in preliminary studies, including polylactide-coglycolide encapsulated vaccine and JE peptides linked to meningococcal preoteosomes.

Vector Control

Field trials of organophosphate larvicides and adulticides have proved effective against vectors of JE. Use of agricultural pesticides in rice-growing areas have also reduced populations of *Culex tritaeniorhynchus*. Integrated programs which include use of chemical larvicides, larvicidal fish, and biological larvicides (*Bacillus thuringiensis*), elimination of aquatic vegetation in irrigation canals, and spraying of residual insecticides in livestock pens have reduced the case incidence in China (240). Emergency epidemic control requires aerial ultralow-volume (ULV) spraying of organophosphate adulticides.

Murray Valley Encephalitis Virus

Epidemics of encephalitis ("Australian X disease") in southern Australia in 1917, 1918, 1922, and 1925 are now believed to have been due to MVE virus. In 1917 and 1918 an infectious agent was recovered by inoculation of monkeys (50), but the virus was not established or characterized. During an outbreak in 1951 the virus was isolated from human brain and was shown to be a flavivirus related to, but distinct from, JE virus (141). *Culex annulirostris* was suspected to be the vector in 1951, and the virus was subsequently isolated from this mosquito in 1960 (121). Epidemics occurred in southeastern Australia in 1956, 1971, and 1974. Small outbreaks also occurred in the Kimberley and Pilbara regions of western Australia in 1971, 1978, 1981, and 1984. The disease was recognized in New Guinea in 1956 (143).

Infectious Agent

Murray Valley encephalitis virus is a member of the JE antigenic complex. Antigenic comparison of five MVE virus strains isolated in Australia and New Guinea by HI, kinetic HI, and plaque-reduction neutralization tests showed four strains to be similar and showed the fifth to have minor but reproducible antigenic differences. Strains from the 1974 epidemic were indistinguishable by standard serologic tests from isolates recovered during previous outbreaks (117).

Mapping of the E glycoprotein of MVE virus with monoclonal antibodies and synthetic peptides has elucidated T- and B-cell epitopes (207,353). The nucleotide sequence of MVE has shown it to be most closely related to JE virus (99,314).

Many vertebrate and mosquito cell culture systems propagate the virus. Plaque assays can be performed in primary chick embryo and continuous lines of pig kidney, monkey kidney, and hamster kidney cells (586). Immune enhancement of virus growth has been demonstrated in primary chick embryo fibroblast cultures mediated by a subpopulation of macrophages having Fc receptors (271). The host range of MVE virus has been reviewed by French (142). Infant mice are highly susceptible by all routes of inoculation. Mice develop clinical resistance to peripheral (but not intracerebral) inoculation between 17 and 28 days of age. Hamsters 6 to 10 weeks old are susceptible to lethal infection by all routes. Monkeys, horses, sheep, and some birds develop encephalitis after intracerebral inoculation. Pigeons and chickens infected subcutaneously develop viremic infections without clinical illness. Rabbits and guinea pigs have subclinical infections after intracerebral and peripheral inoculations. Chicken embryos are highly susceptible and have been used for primary isolation from human cases (141).

Murray Valley encephalitis virus is suspected to cause neurologic disease in horses, but field and experimental studies have failed to conclusively incriminate the virus (348). During epidemics of MVE, there are reports of dogs dying; dogs are susceptible to infection, as shown by a high antibody prevalence, but no evidence has been obtained for a pathogenic role of MVE virus.

Pathogenesis and Pathology

Relatively little is known of the experimental pathogenesis of MVE in laboratory animals. Pathological findings in human cases of fatal encephalitis are similar to those in JE (465).

Clinical Features

The disease begins with a 2- to 5-day prodrome characterized by fever, headache, myalgia, generalized malaise, anorexia, and nausea followed by the appearance of nuchal rigidity and neurologic signs. In infants the disease progresses rapidly, and patients are frequently comatose when first brought to medical attention. Bennett (29) divided patients into three groups on the basis of severity of illness: (a) mild cases with altered level of consciousness and variable neurological abnormalities but without coma or respiratory depression, accompanied by stabilization of neurologic changes within 5 to 10 days; (b) severe cases with coma, paresis, and paralysis, including respiratory and pharyngeal impairment requiring respiratory assistance; and (c) fatal cases with spastic quadriplegia and progressive CNS damage or severe disease with superimposed bacterial infection. Neurologic sequelae occur in up to 40% of the mild cases and in all of the severe cases that recover. Deficits include paraplegia, impaired gait and motor coordination, and intellectual dysfunctions. In one case, a child with *Hemophilus influenzae* meningitis relapsed after an initial response to antibiotics and died of fatal MVE, suggesting that the bacterial infection compromised the blood-brain barrier, permitting neuroinvasion by the virus.

Attempts to associate MVE virus with mild febrile illnesses without neurological signs have failed (117).

Diagnosis

Specific diagnosis depends on isolation from brain tissues of fatal cases or serologic tests. Virus isolation from blood or CSF has not been successful. Isolations from brain have been made in chick embryos and suckling mice. For serodiagnosis, the ELISA, HI, CF, and neutralization tests are useful. Cross-reactions with dengue and with Kunjin virus may confuse interpretation. An epitope-blocking ELISA with MVE monoclonal antibodies proved useful in distinguishing MVE infections from those with other flaviviruses (348). Some patients with encephalitis (presumably MVE) have shown rising antibody titers to Kunjin (118), possibly on the basis of a previous Kunjin infection and the "original antigenic sin" phenomenon. IgM antibodies appear to be quite specific and useful for early diagnosis (592).

Treatment

Treatment is as described for SLE and JE.

Epidemiology

Epidemics have occurred principally in the Murray Valley region of New South Wales and Victoria, involving up to approximately 50 human cases (348). The most notable recent outbreak, in 1974, was unusual in its geographical extent, with patients also infected in east central Queensland, Northern Territory, northern and southeastern South Australia, and the Ord River Basin of Western Australia. Sporadic cases were reported in New Guinea. Outbreaks in Australia occur during the summer (January to May) and appear to follow periods of abnormally high rainfall for 2 consecutive years. Water impoundment and irrigation schemes were responsible for the emergence of MVE in Western Australia in the 1970s. A mathematical model of MVE has provided hypotheses regarding amplification and dissemination of the virus in southern Australia (268).

Population-based incidence data on MVE are not available. Prior to 1974, children were predominantly affected. In the 1974 epidemic, however, 35% of the patients were under 10 years old, and a similar proportion were over 50 years (118). A serologic survey conducted after the 1951 epidemic in various localities in Victoria showed 4.5% to 36% positive CF tests; the rate in children was about one-half that in adults (9). A statistically valid HI antibody survey conducted 8 months after the 1974 epidemic showed 4.5% positive overall. Follow-up surveys indicated no MVE virus activity after 1974, despite a high incidence of human infection with other flaviviruses (208). Murray Valley encephalitis is endemic in the tropical Kimberley area of Western Australia (519). Between 1978 and 1991, there were 26 reported cases of Australian encephalitis in Western Australia, of which 16 occurred in the Kimberley region (338). Of 20 cases serologically diagnosed, 18 had MVE and 2 had Kunjin virus infections. Sixty-five percent of the encephalitis cases were in Aboriginal children and 85% were males.

The principal vector is *Culex annulirostris*, a transient pool breeder. The virus has also been isolated from *Aedes normanensis, Culex tritaeniorhynchus*, and *Culex pipiens. Culex pipiens*, once considered to be a potential vector on epidemiological grounds, has proved to be poorly susceptible or refractory to oral infection in experimental studies, whereas *Culex annulirostris* is a highly efficient vector (266).

Large water birds (such as herons, egrets, and pelicans) appear to be the most important viremic hosts. However, mammals (including rabbits and kangaroos) may contribute significantly to the transmission cycle.

The overwintering mechanism and origin of intermittent epidemics are unknown [for review, see (348)]. Even in tropical areas of Australia, year-round transmission of MVE virus has not been demonstrated. Vertical transmission has been shown experimentally in *Aedes aegypti*.

Molecular Epidemiology

Murray Valley encephalitis viruses analyzed by mapping restriction enzyme digests and by nucleotide sequencing showed a remarkable homogeneity of strains from widely separated areas of Australia over a 23-year period (138,328). This finding suggests continuing gene flow, possibly by widely ranging water bird hosts. Strains from New Guinea differ, suggesting that this area represents a separate focus.

Prevention and Control

No vaccine is available. In areas prone to recurrent epidemics (e.g., the Murray River basin), reduction of *Culex annulirostris* breeding by use of larvicides is practiced. Use of rainfall data and surveillance of virus activity in mosquitoes and sentinel fowl to predict risk of epidemics allows targetcd mosquito control efforts.

Central European and Russian Spring-Summer (Tick-Borne Encephalitis) Viruses

Tick-borne encephalitis was clinically described in the Far Eastern Soviet Union in 1934. In 1937, Silber et al. isolated the causative virus from human brain and demonstrated tick transmission (523). The discase was first recognized in eastern Europe (Czechoslovakia) during an epidemic in 1948, and a virus isolated from a patient was shown to be similar to the Far Eastern virus. Transmission by ingestion of unpasteurized goat's milk occurs in both Russia and Europe. The disease has been recognized in all Central and Eastern European countries and Scandinavia, as well as in France, Italy, Greece, and Albania.

Infectious Agents

The tick-borne encephalitis virus complex consists of 14 antigenically closely related viruses, 8 of which cause human disease (Table 1). Russian spring-summer encephalitis (RSSE) and Central European encephalitis (CEE) viruses are very closely related antigenically and have been considered subtypes of the samc virus. They share about 96% of their amino acids in the E protcin and can be distinguished by means of protein E-specific monoclonal antibodies (239). No significant differencc, however, has been observed in cross-protection experiments when mice were immunized with the European subtype and then challenged with different Europcan and Far-Eastern strains (239). As discussed below, the two subtypes also differ in their tick vectors and clinical expression. Remarkable stability of the CEE genome and antigenic determinants has been demonstrated among strains from different areas of Europe (182, 239,589). The morphology, chemistry, and antigenic composition of TBE complex viruses are similar to those of other flaviviruses (215,223). Although the E protein of TBE virus undergoes specific conformational changes at acidic pH which are associated with a rcduction in infectivity (222), a significant degree of resistance to acid pH in sour milk (170) and gastric juice (431) has been reported. This feature is apparently responsible for oral infections via milk.

Tick-borne encephalitis complex viruses grow in a variety of cell cultures, including pig, bovine, and chick embryo, HeLa, Detroit 6, human amnion, Hep 2, Vero, and primary reptilian and amphibian cells (442), but CPE and plaquing are variable. Chicken embryos and a variety of animals are susceptible to infection. Infant and weanling mice develop fatal encephalitis by all routes of inoculation. Rats, guinea pigs, sheep, monkeys, and swine succumb to encephalitis after intracerebral inoculation. Hamsters are less susceptible to intracerebral and peripheral challenge than mice. Experimental inoculation of wild vertebrate species (including rodents, insectivores, foxes, birds, hares, and bats) results in viremia and antibody formation (168). Cows, goats, and sheep experimentally infected by inoculation or tick bite develop viremia and secrete virus in their milk. Mice can also be infected by the oral route, with subsequent shedding of virus in feces and milk (432).

The Far Eastern virus type (RSSE) is more virulent for sheep and monkeys inoculated intracerebrally than the Western (CFE) virus (609). However, no distinct difference has been noted in pathogenicity for mice or cell cultures (21). Variation has been found between wild virus strains in their ability to produce viremia in a natural host species, *Clethrionomys glareolus* voles (87).

Pathogenesis and Pathology

The pathogenesis in mice was reviewed by Albrecht (5) and does not differ significantly from the general scheme presented earlier. In monkeys the anterior horn cells of the spinal cord and cerebellar cortex appear to be selectively more vulnerable to tick-borne viruses than other neuronal centers (403). An apparently unique feature of members of the TBE virus complex may be their propensity to cause persistent infections in experimental animals (and possibly also in humans). Mice infected with Kyasanur Forest virus may survive for months with paralysis, low titers of virus in the brain, and absence of detectable neutralizing antibodies (439). Hamsters infected with CEE virus de-

velop late CNS lesions, including proliferation and hypertrophy of astrocytes (613), and have detectable viral antigen in tissues for up to 4 months. Louping ill virus persisted in immunosuppressed guinea pigs for 50 days (612). Monkeys infected intranasally or intracerebrally with CEE virus developed a chronic encephalitis with degenerative spongiform lesions and astrocytic proliferation (613). The virus has been isolated from monkey tissues by cocultivation and explant procedures as long as 383 days after inoculation (434). Chronic progressive human encephalitis and seizure disorders (Kozhevnikov's epilepsy) have been associated with RSSE virus on serological grounds (413), and virus has been isolated from the CSF of a patient with a disease clinically resembling amyotrophic lateral sclerosis (396). These observations support the notion that flaviviruses produce persistent infection in sequestered neuronal tissues of human hosts (see Pathogenesis and Pathology, above).

The neuropathology of experimental infection in mice has been reviewed by Vince and Grcevic (571), and the pathology of infection in humans has been reviewed by Zilber and Soloviev (610). Findings are generally similar to those of other flaviviral encephalitides. Gross changes include swelling, congestion, and petechial hemorrhages. Histopathologic alterations include meningeal and perivascular inflammation, neuronal degeneration and necrosis, neuronophagia, and glial nodule formation involving cerebral and cerebellar cortex, brain stem, basal ganglia, and spinal cord. The anterior horn cells of the cervical cord are especially vulnerable, explaining the predominance of lower motor neuron paralyses of the upper extremities seen in many cases.

Clinical Features

The Central European form is milder than RSSE and typically takes a biphasic course. The first phase is an uncharacteristic influenza-like illness with fever (usually not exceeding 38°C), headache, malaise, and myalgia that arises after an incubation period of 3 to 7 days and lasts for about a week. After an asymptomatic period of up to a week, 20% to 30% of those affected develop the second phase of the disease which involves the CNS. It may take the form of aseptic meningitis, meningoencephalitis, meningoencephalomyelitis, or meningoencephaloradiculitis (446,447). The case-fatality rate is 1% to 2%, and the disease in children is less severe than in adults. Meningitis usually resolves completely. However, 10% to 20% of patients with the more severe clinical forms have long-lasting or permanent neuropsychiatric sequelae. These tend to be minor in degree, and motor defects are rare; CSF changes are similar to those in the other flavivirus encephalitides.

The disease in the Far East (RSSE) differs clinically from CEE. Onset of illness is more often gradual than acute, with a prodromal phase including fever, headache, anorexia, nausea, vomiting, and photophobia. These symptoms are followed by stiff neck, sensorial changes, visual disturbances, and variable neurological dysfunction, including paresis, paralysis, sensory loss, and convulsions. In fatal cases, death occurs within the first week after onset. The case-fatality rate is approximately 20% (167) but may be biased by the lack of hospitalization of mild cases. In contrast to CEE, the disease in children is more severe than in adults. Neurologic sequelae occur in 30% to 60% of survivors, especially residual flaccid paralyses of the shoulder girdle and arms.

Diagnosis

Fewer than half of the patients give a history of tick bite. At the time of admission to hospital, i.e., after onset of CNS symptoms, specific antibodies are almost always detectable. Diagnosis is most often made by serological tests, especially the detection of IgM antibodies by ELISA (233, 475). Using sensitive tests, IgM antibodies may be detectable for up to 10 months after disease onset. The virus may be isolated from the blood during the first phase of illness and from brain tissue of patients dying during the early phase of the disease. Infant mice, embryonated eggs, and chick embryo cell cultures have been used for virus isolation; the success rate in any series of patients is, however, less than 10%.

Epidemiology

Tick-borne encephalitis occurs in an endemic pattern over a wide area of Europe and the former Soviet Union, corresponding to the distribution of ixodid tick vectors (Fig. 13). The annual incidence is several thousands of cases, with considerable variation from year to year. In Europe, the highest morbidity has been recorded in Austria, Slovenia, Hungary, and the former Czechoslovakia, with several hundred cases occurring annually in these countries (36,168). In the former Soviet Union, 5,500 cases were reported in 1990. The disease occurs in foci characterized by ecological habitats favorable for ticks, and very little variation in geographic distribution is observed. The intensity of transmission varies from year to year; increases in small mammal populations (the principal hosts for immature ticks), with subsequent migration, are followed within 1 to 2 years by increased tick populations and a higher risk of human infections.

The virus is maintained in nature in a cycle involving ticks and wild vertebrate hosts (Fig. 14). At least ten species of rodents have been implicated as amplifying hosts (167, 168). Insectivores—shrews, moles, hedgehogs—which have relatively stable populations (in contrast to rodents), are believed to be important reservoirs. Large mammals such as goats, sheep, and cattle are important blood-feed-

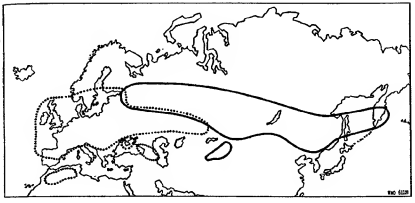


FIG. 13. Distribution of *Ixodes ricinus* (vector of Central European encephalitis and louping ill viruses, *dotted line*) and *Ixodes persulcatus* (vector of Russian spring-summer encephalitis, *solid line*). From Blaskovic, Pucekova, and Kubinyi (36), with permission.

ing hosts for adult ixodid ticks, but have low viremias and are not considered to be important sources of tick infection. The virus is excreted in the milk, however, and human infection may result from consumption of unpasteurized goat or sheep milk or cheese. Small outbreaks, involving all age groups and often in family groups, result from consumption of raw sheep or goat milk or cheese (169). Vertebrate hosts may also be involved in overwintering. Prolonged viremic infections have been demonstrated in hibernating dormice and hedgehogs, bats, and ducks.

Ixodes ricinus and *Ixodes persulcatus* are responsible for transmission in Europe and the Soviet Union, respectively (see Fig. 13). Other tick species, of the genera *Dermacentor* and *Haemaphysalis*, have also been implicated in transmission, especially in areas that do not support *Ixodes* ticks. Pre-imago ticks acquire infection by feeding on small mammals and pass the virus transtadially to the adult stage. Transtadial and transovarial virus transmission has been demonstrated in *Ixodes, Dermacentor*, and *Haemaphysalis*. Considerable losses of virus occur with tick moulting from stage to stage. Approximately 1% of the progeny of an infected female *Ixodes* acquire the virus. Although ticks may serve as a natural reservoir and overwintering mechanism, horizontal transmission between vectors and vertebrates is required for endemnicity. A mathematical model has been described which predicts age-specific infection rates and infection risk in hyperendemic foci (155).

The classical view of the role of vertebrate host in virus amplification has been challenged by laboratory studies showing direct transmission between infected and uninfected ticks cofeeding on rodents in the absence of detectable viremia (308,410). Certain natural hosts of immature tick vectors that are not permissive to viremic infection or that sustain low viremias (such as *Apodemus* mice) may amplify virus transmission in this fashion. Large mammals (goats, sheep, deer) might subserve virus transmission by this mechanism, but are probably often excluded by virtue of immunity. Transmission between cofeeding ticks appears to be activated by a factor present in tick saliva (307). This mechanism for virus maintenance independent of vertebrate animals illustrates the primitive evolutionary status of tick-borne flaviviruses.

In Central Europe, the activity of adult ticks occurs in two peaks (May to June and September to October), that are followed by two peaks of human disease at a 3- to 4-week interval, whereas in Scandinavia only one peak is seen in late summer. Humans are dead-end hosts for the virus. Forestry workers, farmers, and others working in forest or scrub areas are at highest risk. In recent years, however, the infection has increasingly affected people in their leisure and travel activities. An imported case of TBE occurred in a child in Ohio (97).

Before the availability of an effective vaccine and biosafety precautions, laboratory infections with TBE virus were not uncommon (498).

Molecular Epidemiology

Studies on the molecular epidemiology of TBE virus have revealed that strains from Europe (including Scandinavia) isolated over a 26-year period are quite homogeneous, as determined by monoclonal antibody analyses, peptide mapping, competitive RIAs, and comparative sequencing (217,239,589). So far there is no evidence that the virus is subject to major antigenic changes under natural ecological conditions.

Prevention and Control

Using Far-Eastern strains of TBE virus, formalin-inactivated mouse brain vaccines were already used prior to World War II in the former USSR. A live, attenuated heterologous (Langat) vaccine developed in Czechoslovakia was investigated (360) and abandoned. Formalized cell culture-derived vaccines were developed in Russia, and at the present time an unpurified and a partially purified inactivated chick-embryo cell vaccine are in use (130). An experimental vaccine has also been prepared using virus grown in a green monkey kidney cell line (86).

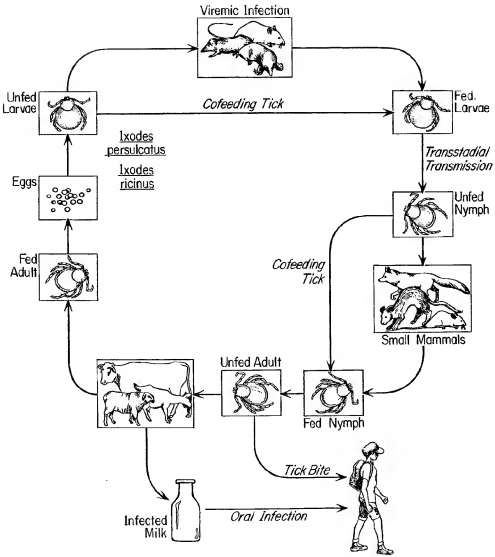


FIG. 14. Generalized transmission cycle of tick-borne flaviviruses, showing hosts for larval, nymphal, and adult ticks. Virus is passed to succeeding tick stages during moulting, as well as transovarially to progeny of adult ticks. Both male and female ticks are involved in transmission. Tick-borne encephalitis virus (and possibly other tick-borne flaviviruses) may be transmitted to unifected ticks cofeeding on a vertebrate host without the requirement for active viremic infection of the host.

In Europe, a partially purified formalin-inactivated vaccine based on an Austrian virus isolate grown in chick embryo cells became commercially available in 1976 (196, 297). This vaccine gave satisfactory seroconversion rates but caused a significant rate of side reactions such as headache, malaise, and fever. These were almost completely eliminated when the vaccine was prepared in a highly purified form by continuous flow zonal ultracentrifugation (216,295). This vaccine is licensed in several European countries, including Austria, Germany, Switzerland, and Sweden. It contains $Al(OH)_3$ as an adjuvant and has an efficacy of about 97% to 98% in the field. In Austria, mass vaccination was initiated in 1981, and this resulted in a dramatic decline of the annual incidence of disease (298). A second European TBE vaccine has more recently been registered in Germany (200,273), and demonstration of efficacy will have to await results from longer-term usage. Vaccination may be warranted in persons living in endemic areas, persons working under high-risk conditions (foresters, woodcutters, farmers, military personnel, laboratory workers), or travelers engaged in high-risk activities (e.g., field work, camping).

For passive immunization, a specific TBE-immunoglobulin is also available in several European countries that can be used for pre- and postexposure prophylaxis. When given within 4 days after tick bite, protective efficacy was estimated to be 60% to 70% (296).

The use of repellents or protective clothing may reduce the risk of tick bite, though this is not a very practical prophylactic measure, especially for persons with a permanent risk of exposure. In the past, widespread use of pesticides, including aerial applications of pesticides, has also been undertaken in some areas in attempts to interrupt transmission.

Other Viruses Causing Encephalitis

Rocio Virus

Rocio virus, a classical "emerging virus," was first isolated from fatal human cases in 1975 during an explosive outbreak of encephalitis on the south coast of Sao Paulo State, Brazil (332,333). The virus is not placed in any of the flavivirus antigenic complexes, but it cross-reacts most closely with members of the JE subgroup, especially Ilheus virus. A plausible explanation for its sudden appearance is a mutation in a strain of Ilheus virus, which has a wide geographic range including Brazil and Argentina. However, at this writing, neither Rocio nor Ilheus virus has been sequenced in an attempt to elucidate the origin of the epidemic.

Rocio virus is pathogenic for infant mice and hamsters and adult mice by all routes of inoculation. Adult hamsters are susceptible to intracerebral inoculation. Guinea pigs survive peripheral inoculation, and newborn chicks develop viremia but rarely show signs of illness. The virus grows to high titer and forms plaques in Vero, BHK, and PS cell cultures. The pathologic changes in humans are typical of other flavivirus infections; the most severely affected structures in seven patients studied were thalamus, dentate nucleus of the cerebellum, hypothalamic nuclei, and substantia nigra (485). In experimentally infected suckling hamsters, Rocio virus produced severe necrosis of myocardium and pancreas (202).

The clinical features in human cases are similar to those of SLE (549). The case-fatality rate in hospitalized patients was 4%. Sequelae, including persistent cerebellar, motor, and neuropsychiatric signs, were noted in 20% of survivors. Diagnosis is by virus isolation from brain tissues of fatal cases or by serology.

Epidemics in coastal Sao Paulo State in 1975 and 1976 resulted in 971 cases, with attack rates of up to 38 per 1,000 inhabitants (247,248,333). The disease appears to have virtually disappeared, suggesting that the "emergent" virus may not have been well adapted for sustained transmission. Since 1976, only one serologically diagnosed case has been reported [a fatal case in an infant in 1980 (249)], although other, clinically suspect cases continue to occur. The highest disease incidence during the outbreak of 1975 to 1976 was young adult males engaged in outdoor work in impoverished agricultural areas. There was no associated disease in domesticated animals. Wild birds are presumed to be viremic hosts in the transmission cycle. A single virus isolation was made from *Psorophora ferox* mosquitoes. *Aedes serratus* and *Aedes scapularis* were implicated as epidemic vectors of Rocio virus on epidemiologic grounds (140). Both *Psorofora ferox* and *Aedes scapularis* were found to experimentally transmit Rocio virus, whereas *Aedes serratus* did not (370). A mouse brain vaccine was evaluated but shown to lack potency (334).

Louping Ill Virus

Louping ill was recognized as a neurological disease of sheep in Scotland during the late 19th century. Isolated in 1929 (436), the virus is a member of the TBE virus complex. By monoclonal antibody analysis, louping ill and Central European encephalitis viruses belong to a distinct western subtype of the TBE virus complex (530). Louping ill virus kills suckling mice by all routes of inoculation; weanling mice, hamsters, and guinea pigs develop fatal encephalitis after intracerebral inoculation. Many cell cultures propagate the virus, and plaque assays may be performed in PS, Vero, and LLC-MK2 cells.

Experimentally infected sheep develop prolonged viremia followed by ataxia, paralysis, and tremors (454). Pathologic changes include diffuse meningoencephalitis with severe chromatolysis and destruction of Purkinje cells, reactive gliosis, and astrocytosis. Virus strain differences have been noted in virulence for sheep. Concurrent *Erlichia* infection (tick-borne fever) and external stress (shipping, cold, etc.) enhance the disease. Though clinical disease has been described in cows, horses, pigs, goats, deer, and dogs, louping ill is predominantly a disease of sheep, which develop a biphasic illness; the first phase is characterized by fever and weakness, followed by a neurologic illness with prominent cerebellar ataxia, hyperexcitability, and progressive paralysis. Epornitics of fatal disease in wild red grouse have been described (455). Experimentally infected red grouse also develop fatal infection and viremias sufficient to infect tick vectors (453).

The human disease was first described in a person with a laboratory infection (464). Thirty-nine human cases have been reported, of which 26 resulted from laboratory exposure (103,518). Of the natural infections, some followed tick bites, whereas others followed direct exposure of abattoir workers, butchers, and veterinarians to sick sheep. Shepherds and crofters are also occupational risk groups. Infection by the oral route has been documented in animals ingesting infected milk or lamb carcasses.

The human disease is biphasic and resembles Central European encephalitis. The incubation period is 4 to 7 days.

The first phase is influenza-like, lasting 2 to 11 days, followed by a remission of 5 to 6 days and then the reappearance of fever and a meningoencephalitic syndrome that last 4 to 10 days. There is leukopenia during the first phase and leukocytosis during the encephalitic phase. No deaths have been reported. The clinical features are reviewed by Smith and Varma (518) and Webb et al. (579). Asymptomatic infections are not infrequent, and abortive, influenza-like illness also occurs. In one laboratory-acquired case, a hemorrhagic diathesis developed, and the disease closely resembled Kyasanur Forest disease. Treatment is symptomatic. Diagnosis is by virus isolation from blood during the first phase of illness, from CSF during the early encephalitic phase, or by serologic tests (HI, CF, neutralization). Antibodies are produced locally in the CNS. The serum/CSF antibody ratio has been employed in human diagnosis (579).

Louping ill virus is distributed in Scotland, northern England, Wales, and Ireland. A virus serologically identical to louping ill virus was recovered from sick sheep in Norway (452). A closely related but distinct agent in Turkey has been associated with louping ill-like disease in sheep [Turkish sheep encephalitis (147)]. The transmission cycle of louping ill involves *Ixodes ricinus* ticks and both sheep and grouse. Control of the disease in sheep is by vaccination, dipping with residual acaricides, and environmental control of ticks. The vaccine now in general use is grown in sheep kidney cell cultures, is formalin-inactivated, and is concentrated by methanol precipitation (55). A killed vaccine for use in laboratory and abattoir workers was once used on an experimental basis (128). Though not tested, it is likely that the TBE vaccine (prepared against the western subtype) would cross-protect against louping ill infection.

Modoc Virus

Modoc virus was first isolated in 1958 from white-footed deer mice (*Peromyscus maniculatus*) collected in Modoc County, California. Isolations have subsequently been made from the same species in Oregon, Montana, and Alberta, Canada. The virus is antigenically related to other flaviviruses recovered from rodents (Cowbone Ridge, Jutiapa, Sal Vieja, San Perlita viruses), and is probably transmitted by contact spread. Virus isolations have been obtained from throat swab speciments of deer mice trapped in the wild. It has not been possible to experimentally infect mosquitoes. The compartmentalization of virus transmission in a rodent-rodent cycle probably accounts for the scarcity of human infections. A single human case of illness attributed to Modoc virus was reported in California in 1966 (451). The patient was a 10-year-old boy who presented with a clinical picture of aseptic meningitis. The infection may have been acquired in Tulare County, where the patient played with deer mice near a rural cabin.

Powassan Virus

Powassan virus was first isolated in 1958 from a fatal case of encephalitis in Ontario (366). It is a member of the TBE virus complex but is more distantly related than other viruses in the complex antigenically (66) and genetically (342). The virus is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes; hamsters and rabbits develop subclinical infections. Experimental encephalitis has been demonstrated in rhesus macaques. Continuous cell lines of monkey kidney origin are useful for virus assay by CPE and plaque formation. Pathologic changes in the brains of mice, monkeys, and humans are typical of other flavivirus infections. Powassan infection is characterized by a variable period of fever and nonspecific symptoms, followed by neurologic signs of meningeal irritation and encephalitis, which are often severe. Serious neurological sequelae have occurred in 35% of the reported cases, and in one these resembled RSSE with shoulder-girdle involvement (93). In another case, the acute disease closely mimicked herpes encephalitis with temporal lobe involvement. In cases reported from the former Soviet Union, a characteristic syndrome with prominent cerebellar signs differentiated the disease from RSSE (316).

The disease occurs in Russia, Canada, and the U.S. In North America, Powassan encephalitis has been reported in Ontario, New York, and Pennsylvania, with a total of twenty cases, eighteen in persons <20 years, and one death (14,555). The majority of cases have been in males, probably reflecting increased outdoor activity and exposure to ticks. Human infections are rare; antibody surveys in the U.S. and Canada have generally shown prevalence rates of 0.5% to 4%. The virus has a much wider geographic distribution than indicated by case reports, and Powassan infection should therefore be suspected in cases of encephalitis throughout the U.S. and Canada. Isolations of Powassan virus have been made as far West as British Columbia and California and as far south as West Virginia, with serological indications for transmission in northern Mexico. Treatment is symptomatic; diagnosis is by serology or virus isolation from brain tissues of fatal cases.

In North America the virus is transmitted in a cycle involving small mammals (principally squirrels and ground hogs) and *Ixodes* ticks, including (a) *Ixodes marxi* and *Ixodes cookei* in the East and (b) *Ixodes spinipalpus* in the western states (14,15). The virus has also been isolated from *Dermacentor andersoni* in South Dakota. *Ixodes dammini* (the vector of Lyme disease) is a competent experimental vector. The widening geographic distribution of *Ixodes dammini* and its role in the spread of Lyme disease may herald an increased risk of human Powassan infection. Serologic surveys and virus isolations have shown infections in wild mammals, including rodents, hares, dogs, skunks, and foxes. In an experimentally infected lactating goat, virus secreted in the milk resulted in infection of the offspring; antibody has been demonstrated in naturally in-

fected goats in New York state, indicating the possibility of milk-borne transmission to humans (598). The virus has been isolated from ixodid ticks (e.g., *Haemaphysalis neumanni*) and from mosquitoes in Russia (315). There is no evidence for mosquito transmission in North America.

Negishi Virus

Negishi virus was isolated from the CSF of a fatal human casein Tokyo in 1948 during an outbreak of JE. The virus is a member of the TBE virus complex (89). Monoclonal antibody analysis and nucleotide sequencing showed that the virus is most closely related to louping ill virus (569). Infant and weaned mice are highly susceptible to peripheral and intracerebral infection. A second fatal case occurred in 1948, and in 1950 a laboratory infection was reported with fever but no neurologic signs (415). Human cases of Negishi encephalitis have been recognized in China.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH FEVER, ARTHRALGIA, AND RASH

Dengue Fever Viruses

The first epidemic of a disease resembling dengue was described in Philadelphia in 1780 by Benjamin Rush. Epidemics were common during the 18th and 19th centuries in North America, the Caribbean, Asia, and Australia (71,515). Transmission by *Aedes aegypti*, first described by Bancroft in 1906, was later proved by Siler et al. (515) and Simmons et al. (516). Ashburn and Craig found a filterable, infectious agent in human blood in 1906; in 1926 and 1931, respectively, Siler et al. (515) and Simmons et al. (516) transmitted the virus to human volunteers and established the incubation period in mosquitoes. The virus was isolated in mice by Sabin and Schlesinger in 1944 (492), and the existence of more than one serotype was established by cross-protection studies in human volunteers (491).

Dengue is a world-wide public health problem; epidemics involving thousands of persons recur in areas of tropical Asia, Africa, Australia, and the Americas where *Aedes aegypti* is present. In the last 20 years, dengue fever and a severe form of the disease described for the first time in 1954, DHF (see Dengue Hemorrhagic Fever, below) have emerged as the most important arthropod-borne viral diseases of humans (176,388). Over 2 billion persons inhabit tropical areas at risk of dengue infection, and up to 100 million cases of dengue fever and 250,000 cases of DHF occur annually on a world-wide basis.

Infectious Agents

Four serologic types (types 1 through 4) are recognized on the basis of plaque reduction neutralization tests (489) and constitute a distinct antigenic complex (66). Dengue types 1 and 3 form a subcomplex defined by monoclonal and polyclonal antibodies (224). Some antigenic heterogeneity is apparent between strains of each type by conventional neutralization tests (488). However, monoclonal antibodies have also shown unexpected relationships at the subcomplex level (e.g., between dengue 1 and 3, 2 and 4, and between dengue and viruses of the JE and TBE complexes) [reviewed in (213,470)]. At a more detailed level, antigen signature analysis of dengue 2 strains by monoclonal radioimmunoassay (RIA) has revealed antigenic variation that correlates with genomic variation demonstrated by RNA oligonucleotide fingerprinting (387). The latter technique has now defined multiple geographic variants of dengue 1 and 2 virus (see section entitled "Epidemiology," below).

Nucleotide sequences of all four dengue types have been reported (110,188,351). Within the dengue virus group, amino acid sequence positional homology of 63% to 68% was found, compared to 44% to 51% between dengue, yellow fever, and WN viruses. Genetic variants of dengue 2 show more than 90% similarity. A genetic relationship was found between dengue 2 virus and Edge Hill virus (37).

Dengue viruses grow in a variety of primary and continuous cell cultures; high yields and demonstration of CPE are difficult to obtain in many systems without adaptation and passage. Cells of human (BSC-1 and HL-CZ, a promonocyte cell line), monkey (LLC-MK2, Vero, primary monkey kidney), hamster (BHK-21), and mosquito origin are most susceptible. Yields of up to 7 to 8 dex/mL, CPE, and plaque formation are obtained under appropriate conditions.

Dengue viruses replicate in the brains of suckling mice and hamsters inoculated intracerebrally (44). However, unadapted virus strains usually produce subclinical infections or only scattered illness with paralysis and death. Neurovirulence for mice and monkeys increases with sequential passage in mouse brain. Guinea pigs, rabbits, cotton rats, adult hamsters, chickens, and lizards are not susceptible to infection (263,499). Embryonated eggs replicate some strains only after repeated passage. Adult mice inoculated with highly adapted dengue types 1 and 2 viruses become infected with or without overt encephalitis (40). Old World and New World monkeys and apes develop subclinical infection and viremia (477,516,591).

Dengue viruses replicate to high titer in *Aedes* spp. and *Taxorhynchites* spp. mosquitoes inoculated intrathoracically or intracerebrally (483).

Pathogenesis and Pathology

Neuroadapted dengue virus produces typical encephalitic lesions, predominantly in the rhinencephalus of infant, weanling, and adult mice. Viral antigen is detectable by immunofluorescence in reticuloendothelial cells of liver,

lymph nodes, and spleens of intraperitoneally infected mice (40). Dengue viral antigens are detectable by Western blotting in suckling mouse brain or liver examined 7 days after intracerebral inoculation (80). Athymic nude mice peripherally infected with adapted dengue virus develop fatal encephalitis and viral antigen in neurons, skeletal muscle, myocardium, and Kupffer cells.

In experimentally infected nonhuman primates, the role of mononuclear phagocytes as principal sites of dengue viral replication has been established by tissue titration and immunofluorescent staining of cells in skin, spleen, lymph nodes, liver, lung, and thymus; *in vitro* infection of monocytes support these findings. Mild inflammatory lesions are found in the brains of monkeys inoculated by the intracranial route.

Classic dengue fever produces self-limited infection in humans. Biopsies of skin lesions have shown swelling of endothelial cells of small vessels, perivascular edema, and infiltration of mononuclear cells.

Dengue viruses multiply in the midgut epithelium, brain, fat body, and salivary glands of mosquitoes (483). No detectable pathologic changes result from infection, and mosquitoes remain infectious for life. Dengue virus replicates in the female mosquito genital tract and may enter the ovum at the time of fertlization, thereby infecting a portion of her progeny (481). Sexual transmission also occurs from male *Aedes* with inherited infections to susceptible females, which may subsequently pass the virus to their progeny (482).

Clinical Features

The clinical manifestations of dengue fever were described by Siler et al. (515), Simmons et al. (516), and Sabin (491). In the typical case, the disease begins abruptly, after a 2- to 7-day incubation period, with high fever, headache, retrobulbar pain, lumbosacral aching pain, conjunctival congestion, and facial flushing. Fever may be sustained for up to 6 to 7 days or may have a biphasic (saddle-back) course. Initial symptoms are followed by generalized myalgia or bone pain that increases in severity, anorexia, nausea, vomiting, weakness, and prostration. The pulse rate may be slow in relation to the fever. Respiratory symptoms (cough, sore throat, and rhinitis) are not uncommon, especially in children. A transient, generalized macular or mottled rash may appear on the first or second day. Coincident with defervescence (day 3 to 5) or shortly thereafter, a secondary rash, maculopapular or morbilliform in nature and nonirritating, appears first on the trunk and then spreads centripetally to the face and limbs but spares the soles and palms. The rash may desquamate. Fever may rise again, creating the second phase of the saddle-back course. Generalized lymphadenopathy, cutaneous hyperesthesia, and altered (metallic) taste sensation may accompany this step of the disease. The peripheral WBC count is depressed with an absolute granulocytopenia, and the platelet count may fall to <100,000/mm^3. Hemorrhagic phenomena are noted in a few cases and include petechiae, epistaxis, intestinal bleeding, menorrhagia, and a positive tourniquet test. Myocarditis and various neurologic disorders have been associated with dengue fever. Neurologic manifestations include encephalopathy and peripheral mononeuropathy, polyneuritis, and Bell's palsy (178,257,422). Central neurologic disorders appear to be more common in DHF than in classic dengue. Reye's syndrome has also been reported to follow dengue infection.

Convalescence may be prolonged, with generalized weakness, depression, bradycardia, and ventricular extrasystoles. Persistent arthritic symptoms are not a feature of dengue and suggest other viral etiologies, including alphavirus (Ross River or chikungunya) infection.

Diagnosis

Exposure by residence or travel in dengue-endemic areas and knowledge about the occurrence of other cases in the community are important clues to the diagnosis. Other infections that clinically may be confused with dengue include influenza, rubella, rubeola, malaria, scrub typhus, leptospirosis, and a variety of other arboviral infections. Rash is a helpful differential sign, but it may be difficult to discern in dark-skinned persons. Epidemic arboviral infections that resemble dengue fever and may be accompanied by rash include chikungunya, o'nyong nyong, WN, Sindbis, Mayaro, and Ross River virus diseases.

Specific diagnosis depends on virus isolation or serologic tests. Virus may be recovered from the blood during the early febrile phase of the illness. Viremia titers in dengue 1, 2, and 3 infections range from barely detectable to 8 dex for 3 to 5 days; titers in patients infected with dengue 4 are approximately 100-fold lower (179). *Toxorhynchites* mosquitoes are sensitive hosts for dengue virus isolation by intrathoracic inoculation; virus can be identified by immunofluorescence staining or CF tests on mosquito tissues within 10 to 14 days after inoculation (284). Larval *Toxorhynchites* inoculated intracerebrally are also useful for primary virus isolation. *Toxorhynchites amboinensis* (TRA-284), *Aedes albopictus* (C6/36), and *Aedes pseudoscutellaris* (AP-61) cell lines are now widely used for primary dengue virus isolation (293). The TRA-284 line adapted to serum-free medium provides the most sensitive assay. Syncytial CPE may be present, but it is an unreliable marker of infection; moreover, cultures must be examined by immunofluorescence (IF) to detect virus. Monoclonal antibodies are used for type-specific identification by IF. These techniques allow isolation and identification in as short a period as 2 days, depending upon titer of virus in the test sample.

Direct detection of dengue viral antigen in human serum has been reported by use of countercurrent immunoelectrophoresis and by monoclonal RIA. Rapid diagnosis has

been achieved by immunocytochemical staining of peripheral blood mononuclear cells obtained during the acute phase of illness (399), and this method is more sensitive than virus isolation. Reverse transcriptase-polymerase chain reaction (RT-PCR) has been applied to the rapid diagnosis of dengue infections, using serotype-specific primers and analysis of amplified sequences by agarose gel electrophoresis or hybridization with serotype-specific digoxigenin-labeled probes (112,310). This technique has permitted detection of viremia levels <2 dex/mL.

Serologic diagnosis depends on the demonstration of a fourfold or greater rise (or fall) in antibodies by the HI, CF, or neutralization test. It is frequently difficult to establish the specific infecting serotype because of cross-reactions, especially in individuals with preexisting heterologous immunity. In the case of sequential dengue infections, the antibody response to the initial infecting virus type may exceed that to the current infecting type ("original antigenic sin") (194), but this is not a uniformly reliable method for retrospectively determining the identity of an earlier infection (292). The plaque-reduction neutralization (N) test is more specific than other tests. During the convalescent period after a sequential infection, cross-reactions make specific diagnosis difficult, but after approximately 6 months, the presence of neutralizing antibodies to multiple dengue serotypes is a reliable marker of prior infection those serotypes. Epitope-blocking immunoassays employing monoclonal antibodies provide diagnostic specificity similar to the N test (61). The IgM antibody-capture ELISA is the favored assay for serological diagnosis. IgM antibodies appear shortly after defervescence and wane after 1 to 2 months (245).

Because of the association between sequential infection and DHF, it is important to distinguish primary from secondary dengue infections. The HI test has been used to classify dengue infections. Secondary infections are characterized by the presence of HI antibodies in the acute phase sample and by high titers (>1,280) in convalescent sera. The ratio of IgM and IgG antibodies determined by ELISA is useful for distinguishing primary from secondary infections (245,291); in primary infections the IgM/IgG ratio (expressed in OD units) in acute sera or convalescent sera obtained during the first month after onset generally exceeds 1.5, whereas secondary infections are characterized by an excess of IgG. Western blotting of sera from patients with primary dengue infections reveals antibodies against the E glycoprotein and the NS3 and NS5 nonstructural proteins, while sera with high titers due to secondary infections reveal a broader array of viral protein bands (79,80).

Treatment

Treatment is supportive and includes bed rest, antipyretics, and analgesics. In case of dehydration, fluid and electrolyte replacement are used in addition.

Epidemiology

Dengue virus is transmitted in a cycle involving humans and mosquitoes, *Aedes aegypti* being the most important vector (Fig. 15). Dengue occurs principally in tropical areas of Asia, Oceania, Africa, Australia, and the Americas. Temperate areas within the range of *Aedes aegypti* are susceptible to summertime introduction and spread of the virus. In areas having year-round vector activity and large human populations, one or more dengue virus types may be maintained endemically. Elsewhere, especially in small, insular populations, epidemics result from the introduction of a new type.

Protection against homotypic reinfection is complete and probably lifelong, but cross-protection between dengue types lasts less than 12 weeks (491). Consequently, it is possible to sustain multiple, sequential infections. In general, however, most clinically overt illness probably occurs during primary or secondary infections. Clinically apparent tertiary infections do occur, but disease upon infection with a fourth serotype is nearly always subclinical. Experimental infection in nonhuman primates has also shown incomplete cross-protection between pairs of dengue viruses, and sequential infection with three or four dengue types is required to achieve complete protection. The background of homotypic immunity in a human population group determines the incidence and age distribution of infections. In virgin-soil outbreaks, dengue attack rates are generally similar among age groups. In some outbreaks, a higher incidence of dengue fever has been found in females (11,261), probably indicating risk of infection around the home by the domestic, daytime biting vector, *Aedes aegypti*.

Vector density and factors determining exposure to infected female mosquito vectors are important determinants of the rate of dengue virus transmission. The domestic habits of the principal vector, *Aedes aegypti*, assure that infection occurs in and around human habitations. Inhabitants of screened houses are at significantly lower risk of epidemic dengue (274). Where dengue is transmitted, it is not uncommon to find 10 to 20 female *Aedes aegypti* per room, of which 5% to 10% may be infected (243). *Aedes aegypti* commonly exhibit interrupted feeding behavior, and most females feed on blood multiple times between egg-layings; these factors contribute to the rapid transmission of dengue virus and the explosive nature of dengue epidemics.

The world-wide incidence of dengue has increased dramatically in the period following World War II, due to expanding urban human populations and a coincident increase in *Aedes aegypti* density, as well as the advent of air travel and rapid movement of viremic persons. These changes are underscored in the Americas (176,388), where the frequency of epidemics has increased dramatically and multiple dengue serotypes have been introduced. In the past 15 years, the continent of South America was reinvaded by *Aedes aegypti* (Fig. 16), with the result that large dengue

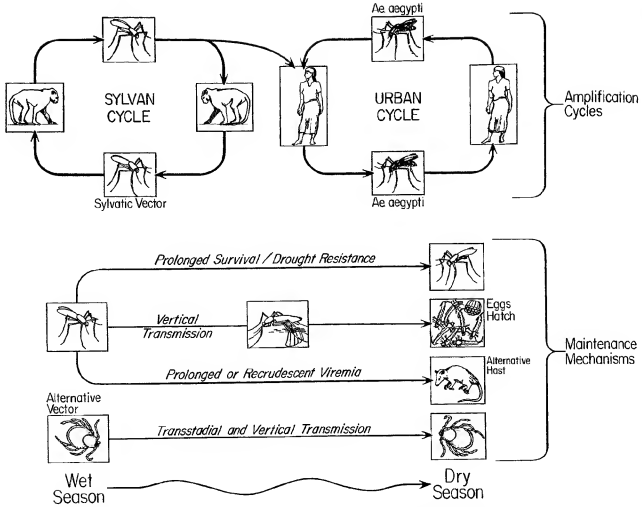


FIG. 15. Transmission cycles of yellow fever and dengue viruses. These viruses have enzootic maintenance cycles involving tree-hole breeding *Aedes* spp. vectors and nonhuman primates. Dengue viruses are transmitted principally between humans and *Aedes aegypti* that breed in domestic and peridomestic water containers. A sylvatic cycle has been documented in Southeast Asia and West Africa, but it is presently uncertain to what extent this cycle contributes to human infections. In the case of yellow fever, sylvatic transmision is widespread throughout the distribution of the virus. In tropical America, human yellow fever cases derive from contact with forest mosquito vectors, and no urban (*Aedes aegypti*-borne) yellow fever has occurred for over 50 years. In Africa, sylvatic vectors are responsible for monkey-monkey and interhuman virus transmission, and there is frequent involvement of *Aedes aegypti* in urban and dry savannah regions.

outbreaks, some involving >1 million persons, occurred in immunologically naive populations. Attack rates in outbreaks in the Americas have ranged from 20% to 90% (51, 129,135). In many areas, dengue has become established in endemic patterns of infection. In Puerto Rico, for example, dengue serotypes 1, 2, and 4 have circulated continuously since 1985.

In hyperendemic areas of Southeast Asia, over 50% of children experience infection with one or more dengue serotypes by age 7. In tropical areas, epidemics tend to occur during the monsoon or rainy season. Although increased rainfall results in expansion of vector mosquito breeding, human disease incidence does not correlate closely with vector population density. Other factors (in particular, increased temperature, which shortens the extrinsic incubation time of dengue virus in the vector) appear to be more important (577).

In the U.S., most cases of dengue fever have been acquired abroad. Between 1980 and 1989; 1,457 cases of clinical dengue were reported in the U.S., and 276 were confirmed by laboratory tests. Two small outbreaks involving spread of the virus from Mexico and secondary transmission within southern Texas occurred in 1980 and 1986 (176,177).

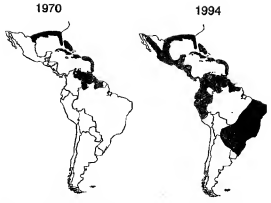


FIG. 16. Distribution of *Aedes aegypti* in the Americas in 1970 and 1994. Reinvasion of the South American continent by Aedes aegypti occurred in the late 1970s and 1980s due to collapse of vector control programs, the increase in breeding sites due to urbanization, and other factors. The result has been the emergence of epidemic dengue and dengue hemorrhagic fever in the continent (see also Fig. 21), and an increased risk of urban outbreaks of yellow fever. From Gubler and Trent (176), with permission.

In addition to *Aedes aegypti*, other species, including *Aedes albopictus*, *Aedes polynesiensis*, and *Aedes scutellaris*, play a role in rural and insular areas of Asia and the Pacific. *Aedes albopictus* was introduced from Asia into the U.S. and Brazil in 1985; an isolation of dengue virus has been made from this species in Brazil, but it has not yet been clearly implicated in epidemic dengue transmission (176,388). *Aedes aegypti* breeds in artificial containers with clean water in and around human habitations and bites principally during the daytime. In addition to biologic transmission, mechanical spread by *Aedes aegypti* and other mosquitoes may occur. Geographic variation exists between strains of *Aedes aegypti* and *Aedes albopictus* in terms of their vector efficiency (susceptibility to oral infection), but the epidemiologic importance of this variation has not been clearly established (175). In general, *Aedes aegypti* strains display low susceptibility to oral infection. This requires that virus titers in the blood of human hosts be high, exceeding 5 dex/mL, in order for infection and transmission to be sustained. Thus, the vector serves as an important selection mechanism or biological filter for maintaining virus virulence at a high level, since only virus strains that replicate efficiently in humans and produce high viremias are transmissible by this mosquito.

Zoonotic cycles of dengue virus transmission involving monkeys and forest *Aedes* spp. have been documented in Malaysia (487) and West Africa (95,467) (Fig. 15). The vector in Malaysia is *Aedes niveus*; the species implicated in West Africa are *Aedes furcifer, Aedes taylori, Aedes luteocephalus, Aedes opok*, and *Aedes africanus*. The possibility of a forest cycle in South America deserves study.

The maintenance of dengue viruses between epidemics has not been clearly defined, especially in rural areas with relatively sparse human populations. An alternative mechanism to year-round horizontal human-mosquito-human transmission such as vertical transmission in *Aedes* vectors, must be considered. Similar considerations apply to the maintenance of the sylvatic cycle over the dry season. Experimental studies have documented vertical transmission of dengue virus in *Aedes*, and the virus has been isolated from field-collected larvae of *Aedes aegypti* and males of *Aedes furcifer-taylori* in West Africa [for review, see (174,175)].

Molecular Epidemiology

Nucleotide sequencing of the entire E gene has replaced the traditional techniques of RNA fingerprinting, RNA-DNA hybridization, antigenic analysis and shortRNA oligonucleotide fingerprinting, RNA-DNA hybridization, and limited nucleotide sequencing of short regions of the genome as tools for determining the origin and spread of dengue epidemics (113,176,387,463,551). Phylogenetic and nucleotide sequence signature patterns of the E gene permit classification of each dengue serotype into a number of E-genotypes, which generally correlate with geographic origin (Table 5). However, the current classification will expand as more strains of each dengue serotype are examined. Within a single geographic area, genetic changes in the virus population may be found over time (574), with the appearance of new variants by mutation and selection as well as by introduction from afar. Inconsistencies in the geographic classification, e.g., the finding of sequence homology between a virus genotype from one region and a strain from a widely separated region, imply that a new strain has been introduced by viremic humans or possibly by virus-infected mosquitoes or mosquito ova. Thus, genotypic classification has been useful as a tool to determine the origin and spread of epidemics. Two distinct genotypes of dengue 1 virus (E-genotypes I and II, Table 5) coexist in Thailand. Genotype II has been recovered in Africa. In 1977 it was introduced into the American region, where it caused a pandemic. The genotype has persisted in the Americas, and its distribution has expanded to include most Latin American countries except Argenina and Chile. Another example of a stably transmitted virus is the E-genotype I of dengue 2 virus. This genotype, represented by the New Guinea C strain, the prototype dengue 2 virus, isolated in 1944, has persisted to the present day in Asia and was introduced into the Americas in 1981, causing the first epidemic of DHF in Cuba (185). This genotype now coexists in multiple areas with E-genotype III, which had been present in the Caribbean since at least 1969. Similarity between the Caribbean E-genotype III strains and dengue 2 strains isolated in the South Pacific between 1971 and 1976 indicated a potential route of

TABLE 5. *Classification of dengue viruses into E-genotypes.*

Virus	Genotype	Geographic distribution
Dengue 1	I	Japan (single isolate, 1943), Hawaii (single isolate, 1944), Thailand, Indonesia, Philippines, Taiwan, South Pacific
	II	Thailand, Malaysia, Burma, West Africa, Americas (introduced in 1977)
Dengue 2	I	New Guinea (prototype), Sri Lanka (prior to 1969), Philippines, Taiwan, Thailand, Malaysia, SE China, Americas ("Jamaica topotype" introduced in 1981)
	II	Seychelles, Sri Lanka, Africa (introduced to East Africa in 1980–1981), Indonesia, Saudi Arabia
	III	India, South Pacific, Americas ("Puerto Rican topotype" introduced in 1969)
	IV	West Africa (sylvan cycle)
Dengue 3	I	Philippines, Indonesia, Malaysia, South Pacific (1980s)
	II	Thailand
	III	Sri Lanka, India, Africa
	IV	South Pacific (1960s), Americas (1960s, 1970s)
Dengue 4	I	Indonesia, South Pacific, Americas
	II	Thailand, Philippines

Genotypes are defined on the basis of phylogenetic relationships of the E gene nucleotide sequence. The classification is a synthesis of several studies [see (77,176,311,463)], and will change as sequences of other strains are reported and evolution of dengue virus genomes continues. The geographic distribution reflects virus importations and movements, and patterns of epidemic spread (see examples in text).

spread. The dengue type 4 virus, circulating in endemic/epidemic pattern in the Americas since 1981, is closely related to strains from Niue Island and the Gilbert Islands, again indicating virus transfer between the Americas and Oceania. Comparison of dengue type 2 strains isolated in the 1980s in Africa demonstrated that enzootic strains associated with sylvatic vectors and nonhuman primates in West Africa were genetically distinct from strains causing human epidemics. The West African epidemic strains were genetically related to isolates from Indonesia, Sri Lanka, the Seychelle Islands, and Somalia, suggesting a route of spread across the Indian Ocean to East Africa and thence to West Africa; the epidemics thus arose by introduction by viremic travelers rather than from a local jungle cycle (176,463).

Genetic analysis of strains has also suggested that dengue viruses vary in virulence (see "Dengue Hemorrhagic Fever").

Prevention and Control

The development of a safe effective dengue vaccine is a high priority of the World Health Organization, health ministries in some affected countries, the U.S. military, and at least one major pharmaceutical company, but has been an elusive goal. Classical live vaccines prepared by serial passage of dengue types 1 and 2 in mouse brain were tested in humans 50 years ago (491,596) and found to be attenuated and immunogenic; however, the unacceptable substrate for these vaccines and the observation that neurovirulence of dengue viruses increased with continued mouse brain passage led efforts in a different direction. Propagation of mouse-adapted virus in chicken embryos (a technique similar to that used to develop yellow fever 17D vaccine) was successful, and the passaged virus induced abortive illness in human subjects (500). Although promising at the time, this approach was not pursued into development. In the 1970s, efforts to produce a live, attenuated vaccine by serial passage and cloning in cell culture (fetal rhesus lung or primary dog kidney) were initiated by the U.S. Army. By this time, it was clear that a combination vaccine that simultaneously induced protective immunity against all four serotypes would be required in order to avoid sensitizing the vaccinee to more severe disease (DHF). Candidate vaccines developed against the four serotypes had various markers of attenuation (small plaque size, temperature sensitivity, reduced viremia in monkeys) and were eventually tested in humans. All were found to be either over-attenuated and poorly immunogenic or to induce denguelike illness of varying severity. The dengue type 2 vaccine (PR-159/S-1) was satisfactorily attenuated (509), but elicited durable immunity only in subjects who had been immunized previously with yellow fever 17D, presumably on the basis of antibody-dependent enhancement (19).The experience gained in these studies suggested that *in vitro* (and even *in vivo* nonhuman primate) markers of attenuation were not reproducibly predictive of the response in humans, and current efforts are thus focused on stepwise testing of selected passage levels in small numbers of human volunteers.

Live, attenuated, small-plaque, temperature-sensitive dengue vaccines produced by serial passage in primary dog kidney cells without biological cloning were developed in Thailand and have shown promise in human trials (32,33). The candidate vaccines have been administered to nonflavivirus immunes first as univalent and ultimately as tetravalent vaccines. Studies are currently underway in adults with prior dengue and JE virus immunity and in seronegative children. The results indicate that the quadrivalent vaccine is safe and immunogenic, and that prior flavivirus expo-

sure enhances the immune response to dengue. The precise dose requirements for each of the four serotype components of the vaccine mixture are still being refined, and it appears that children require significantly lower doses than adults to achieve adequate immunogenicity. Commercial production (by Pasteur-Mérieux Serums et Vaccins, Lyons) of the live dengue vaccine is expected in the near future.

Various strategies are also being explored toward the development of genetically engineered vaccines. Underlying assumptions of this work are: the immunity to protein E (and possibly also NS1) is required for durable protection; both humoral antibodies (especially neutralizing anti-E antibodies) and cytotoxic T-lymphocyte responses mediate protection; immunization with dengue virus is serotype specific (166) and, therefore, simultateous immunization against all four serotypes is required. The major approaches are listed below, with selected examples.

1. Recombinant subunit vaccines: expression of dengue structural proteins in eucaryotic systems has yielded immunogenic and protective antigens. Recombinant baculovirus expressing a fusion protein of E lacking the C-terminal transmembrane domain with MalE. The fusion protein is secreted as a soluble product by *Spodoptera frugiperda* cells and is readily purified by maltodextrin affinity chromatography. The recombinant truncated E protein elicited neutralizing antibodies and protection in mice (107). In another study, a dengue type 1 E gene expressed in baculovirus also induced neutralizing antibodics and was protective (444). However, similar recombinant proteins have not always resulted in retention of conformational dengue epitopes required for neutralization (134). Immunization of monkeys with recombinant baculovirus-expressed structural and nonstructural proteins elicited binding antibodies, but poor neutralizing antibody responses and poor protection against challenge (125).

2. Production of recombinant noninfectious subviral particles: cells infected with recombinant vaccinia virus expressing the prM-E or prM-E-NS1 of JE virus were found to secrete the encoded proteins, which self-assemble into noninfectious, viruslike particles (352). The subviral particles appear to be highly immunogenic and protective (278). A similar approach to the production of dengue particles is now under investigation. A serious concern for the use of subunit vaccines (methods 1 and 2) is their ability to elicit sustained humoral immunity, since waning antibody levels may precipitate aggravated disease due to ADE. The ability of subunit vaccines to induce protective responses based on cellular immunity is uncertain. Given classical concepts of antigen processing and presentation, such vaccines should induce predominantly CD4+ T-cell responses, and would not be expected to elicit CD8+ MHC class I restricted Tc cells that may play a role in clearance of infection. Nevertheless, protection has been demonstrated in mice immunized with recombinant structural proteins in the absence of detectable neutralizing antibodies (134), suggesting that linear T-cell epitopes inducing CD4+/CD8- MHC Class II restricted Tc cells (304,305) may be responsible.

3. Live attenuated, genetically-engineered vaccines: full-length genomic cDNA of a Caribbean strain of dengue type 4 virus has been used to transcribe infectious RNA (309) and to produce stable progeny virus. The cDNA can be genetically manipulated to introduce deletions or substitutions. Mutations introduced in the 3′ noncoding region and at the NS1/NS2a cleavage site resulted in reduced replication of dengue 4 virus, suggesting that the virus had become attenuated. Other potential target sites of the genome for the introduction of attenuating mutations/deletions are under investigation. The infectious clone has been used to construct intertypic chimeric viruses in which the C-prM-E genes of dengue type 1 or 2 or the prM-E genes of dengue 2 were inserted into the dengue type 4 genomic framework (48). In animals, the chimeric viruses elicited protective homotypic immunity to the insert. Similar chimeras have been constructed using the prM-E structural genes of TBE virus in the dengue 4 genomic framework (430). Work is underway in several laboratories to use a similar approach, but with dengue virus structural genes inserted into the backbone of yellow fever 17D virus. While this approach shows considerable promise as a means of preparing rationally designed live vaccines, the genetic changes required to achieve the appropriate level of attenuation and immunogenicity in such constructs remain to be defined.

4. Live, vectored vaccines: various pro- and eukaryotic vectors expressing dengue genes are under investigation, including *Salmonella*, vaccinia, rubella virus, and adenoviruses. The highly attenuated NYVAC vaccinia strain expressing JE prM-E-NS1 was protective in a clinically-relevant host (swine) (279), and immunization of mice with recombinant vaccinia expressing dengue type 1 prM-E genes elicited protective responses (47,139). The efficacy of these vaccines may depend upon the expression of subviral particles [see 2. above] during *in vivo* replication of the recombinant vaccinia.

Until the successful development and implementation of dengue vaccines, prevention of epidemics will continue to rely on reduction or eradication of *Aedes aegypti* by breeding site elimination, use of larvicides, and perifocal spraying of insecticides. Eradication achieved 20 years ago in many countries of Latin America has now been reversed by repeated reinfestations, economic development with attendant expansion of *Aedes aegypti* breeding, reduction in program support, and insecticide resistance (Fig. 16).

For the emergency control of epidemics, it is necessary to interrupt transmission by killing infected adult female *Aedes aegypti*. Ultralow-volume aerial or ground applications of organophosphate insecticides have been used with variable success. Recent field studies in the Caribbean and

South America suggest that ULV applications are relatively ineffective and do not suppress vector populations long enough to interrupt transmission, mainly because of poor indoor penetration of insecticide (404).

West Nile Fever Virus

Infectious Agent

West Nile virus is a member of the antigenic complex of flaviviruses which includes MVE, SLE, and JE viruses. The structures of the WN virus genome and glycoprotein spike have been partially analyzed (409). Kunjin virus (another agent in the virus complex) is more closely related to WN than to other members of the complex (92,586). A high degree of cross-protection was found in hamsters immunized with JE or SLE viruses and challenged peripherally with WN (198). Monkeys immunized with WN were partially protected from lethal intranasal JE virus challenge, whereas JE-immune animals were fully protected against WN challenge (163).

There is a considerable body of research on antigenic variation and restriction length polymorphism analysis of WN virus. Strains from Africa, Europe, the former Soviet Union, and the Middle East as far east as Pakistan form one group distinct from strains isolated in India and the Far East (146,440). Other studies have shown considerable heterogeneity among strains isolated within a single region (30). In Madagascar, for example, monoclonal antibody analysis identified five antigenic variants, of which four represented the African-European antigenic group and one resembled the India-Far East group (394). A Madagascan strain was found to be distinct from other WN viruses at the nucleotide sequence level (438). The results of antigenic and genomic analyses may be interpreted to indicate intercontinental exchange of WN virus strains by migrating birds, but with local segregation of distinct genotypic variants in some areas. In the Central African Republic, for example, where human disease has been characterized by hepatitis, virus strains recovered from humans, mosquitoes, and ticks differed from the Egyptian prototype and from local avian isolates by monoclonal antibody analysis and restriction digest profiles (355). A mosquito isolate from the Central African Republic was placed (together with an East African strain) in a distinct group based on nucleotide sequence (438). The results suggest that the distinctive human disease pattern in this part of Africa may be caused by a local WN variant with a nonavian transmission cycle.

West Nile virus grows and produces CPE or plaques in a wide variety of cell cultures (including primary chick and duck embryo), as well as in continuous lines of human, primate, swine, rodent, and amphibian origin. It multiplies in *Aedes aegypti* and *Drosophila* cells and produces CPE in *Aedes albopictus* cells.

Mice and hamsters of all ages are susceptible to lethal infection by the intracerebral route. Resistance to peripheral routes of inoculation develops with age; some virus strains are pathogenic for adult animals. Lethal oral infection of adult mice has been described. Hamsters transmit virus to their young via the milk. Rabbits, guinea pigs, and cotton rats develop antibodies without overt illness by all inoculation routes; rats succumb to intracerebral infection only. Rhesus and bonnet macaques develop fatal encephalitis after intracerebral or intranasal inoculation (163,401). Almost all birds tested develop viremia, including wild species, chickens, and pigeons; encephalitis and death may occur but are rare (362,543). The developing chick embryo is highly susceptible to the virus. With one exception (namely, *Arvicanthus abyssinicus*), wild African rodents do not develop viremia.

Sporadic cases of naturally acquired WN encephalitis have been reported in horses in Egypt and France (181); however, low-level viremia, antibody production, and absence of clinical illness are the rule. Bovine species do not develop viremia after experimental inoculation, but antibodies in cattle are prevalent. Dogs are susceptible to infection, and some develop mild illness, but low viremia levels appear to preclude a major role in transmission cycles (35).

Strain variation exists in the pathogenicity of WN virus for cell cultures, mice, pigeons, and lemurs (566). Experimental infections in various arthropods have been reported [for review, see (211)]. *Culex univittatus*, the principal vector in Africa, is a highly efficient vector. The virus has been found to infect soft and hard ticks under natural and experimental conditions.

Pathogenesis and Pathology

The pathogenesis of WN virus is similar to that of other flaviviruses (289,343,402). West Nile virus is reported to produce persistent infection and a subacute inflammatory-degenerative process in the CNS of monkeys (433).

Pathologic observations in humans are limited to a very small number of patients with fatal encephalitis and showed lesions of diffuse inflammation and neuronal degeneration. Autopsies performed on patients who died within 4 weeks after inoculation of WN virus (used as an experimental treatment for cancer) resulted in WN virus isolation from spleen, lymph nodes, liver, and lungs (525,526), a distribution similar to that described in laboratory animals.

Clinical Features

The incubation period is 1 to 6 days. The typical case is quite mild, characterized by fever, headache, backache, generalized myalgia, and anorexia. The course of fever may be biphasic. Rash occurs in approximately half of the cases; onset of rash is either during the febrile phase or at the end of it. The rash is roseolar or maculopapular, is nonirritat-

ing, and principally involves the chest, back, and upper extremities. Rash may persist for up to a week and resolves without desquamation. Generalized lymphadenopathy is a common finding. Pharyngitis and gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain) may occur. The disease runs its course in 3 to 6 days, followed by rapid recovery (344). Children generally experience milder illness than adults. Infection may also result in aseptic meningitis or meningoencephalitis in a small proportion of patients, especially in the elderly. Other neurologic presentations include anterior myelitis resembling poliomyelitis and encephalopolyradiculitis (58,164). In an outbreak of encephalitis in 12 of 49 aged persons who acquired the infection in Israel, four patients died. Three cases of encephalitis in young people were described by Flatau et al. (137); one patient had papillitis. Eleven percent of cancer patients inoculated with the prototype (Egypt 101) strain showed clinical signs of encephalitis (526), an incidence much higher than expected from experience in naturally acquired infection, probably due to the altered susceptibility and poor immune responsiveness of the patient population. Nonneurologic, rare complications include myocarditis (4) and pancreatitis (427). In the Central African Republic, WN virus has been responsible for cases of hepatitis, including fatal disease resembling yellow fever (152).

Clinical laboratory findings include leukopenia and, in cases with CNS signs, CSF pleocytosis and elevated protein.

Inapparent and very mild infections are common. In the series of cancer patients intentionally inoculated by Southam and Moore (526), 89% of 78 infected patients had no clinical signs or symptoms other than fever; in 27%, fever never exceeded 1°F.

Diagnosis

On clinical grounds, WN fever resembles dengue and other denguelike fevers. Other manifestations include meningoencephalitis and (in Central Africa) hepatitis. Unlike SLE, JE, and MVE viruses, WN virus can be isolated from the blood of as many as 38% of patients (77% when the specimen is taken on the first day of illness) (154). In persons naturally infected, viremia was detectable up to 5 days after onset; titers were low (maximum titer 3.3 dex/mL). In cancer patients inoculated with the Egypt 101 strain, viremias were more prolonged and of higher titer (526). Viremia has been demonstrated by chance during the incubation period. The reverse passive hemagglutination test has been used to detect viremia in the blood of nestling birds. This technique as well as immunoassays have not been evaluated for detection of virus in human clinical specimens.

Serologic diagnosis is possible using any of the usual tests; cross-reactions with heterologous flaviviruses complicate the interpretation. In addition to standard serologic tests, the indirect IF and ELISA tests are applicable to the diagnosis of WN infection. Radial hemolysis-in-gel has been used for the detection of IgG antibodies, has a similar sensitivity to the HI test, and has the advantage that there is no need to remove nonspecific inhibitors.

Treatment

Treatment is supportive.

Epidemiology

West Nile virus is widely distributed throughout Africa, the Middle East, parts of Europe and the USSR, India, and Indonesia. During the 1950s, classic studies on the epidemiology of WN virus in Egypt and the Sudan revealed that human infections were extremely common in the Nile Delta (543), where 22% of children and 61% of young adults were immune. A survey conducted 10 to 15 years later showed that the infection prevalence in adults had decreased somewhat (to 50%) (101). In a study conducted in 1968, 14.6% of febrile children attending the fever hospital in Alexandria were diagnosed as having WN infections (372). A current survey showed that 6% of schoolchildren and 40% of young adults had been infected (96). The hyperendemic virus circulation has precluded sharp epidemics but places a high burden of infection on childhood populations, which experience largely unrecognized and clinically undifferentiated febrile disease. The risk of epidemics may increase (especially in areas such as Cairo) as endemic transmission declines and a larger segment of the adult population becomes susceptible.

Summertime epidemics of WN fever were recognized as early as 1950 in Israel and recurred there at frequent intervals during the 1950s (344). These epidemics involved hundreds of recognized cases, but the true incidence was undoubtedly much higher, and attack rates of over 60% were reported in some localities. The epidemics were the result of amplified virus transmission and spill-over to a human population with a low background of immunity.

In an area of South Africa with a relatively low background of immunity to WN virus (13% to 20%), an outbreak in 1974 resulted in infection of 55% of the population. The epidemic involved an area of 2,500 km^2 of central and northern Cape Province. Hundreds to thousands of clinical cases occurred, but they were mild and without any recognized cases of encephalitis (364). Few clinical cases have been reported from West and Central Africa (152,550). In the central highlands of Madagascar, a high annual incidence of WN virus infection (15%) has been documented, and the virus is suspected to be responsible for grippelike illness in the population (394,395). A small outbreak of WN virus infection occurred between 1962 and 1964 in the Camargue region of France, in which 13 cases were documented, some with encephalitic complications (420).

Infections in tropical Asia are frequent, and the virus appears to be hyperendemic in many areas.

The incidence of CNS infection has not been clearly defined, but this complication appears to be rare. Cases have been described in Israel, India, France, and Egypt. In Egypt, 4 of 133 patients with aseptic meningitis or encephalitis admitted to one hospital between 1966 and 1968 were shown to have WN infection (2).

West Nile virus has been isolated from various mosquito species; *Culex univittatus* and *Culex pipiens molestus* appear to be the most important vectors in Africa and the Middle East (211). The virus has been isolated from an extensive list of *Culex*, *Aedes*, *Anopheles*, *Mimomyia*, and *Mansonia* mosquitoes in Africa. *Culex tritaeniorhynchus* is an important vector in tropical Asia. Isolations of the virus has been made from ticks, especially in bird rookeries in the former Soviet Union. There have been numerous isolations from wild birds in many areas, and high antibody rates in birds have been reported in Israel, Egypt, and South Africa. Birds sustain high viremia after experimental infection, and are the only known amplifying host (362). West Nile virus has been isolated from a camel and a grass mouse in Nigeria, and a frugivorous bat in India (211,263). Bats have also been implicated as possible hosts in Madagascar. Humans and horses susceptible to clinical infections are incidental hosts and are not thought to play a role in the mosquito transmission cycle. The mechanism of virus survival over the winter or dry season has not been elucidated. Vertical transmission of WN virus in *Culex* and *Aedes* species has been demonstrated under laboratory conditions (25). Transtadial transmission by ticks may play a significant role in virus maintenance. It will be of interest to determine whether the phenomena of nonviremic, saliva-enhanced transmission of WN virus occurs between cofeeding ticks (410).

Prevention and Control

There is no vaccine. In the event of epidemics, use of space sprays to kill infected adult mosquitoes may be warranted.

Other Viruses

In addition to WN and dengue, other flaviviruses cause human disease. These agents are not of major public health importance but must be considered in the differential diagnosis of febrile illness in persons inhabiting or traveling to endemic areas.

Banzi Virus

The Banzi virus was first isolated from the blood of a febrile child in South Africa in 1956 (522). It has been associated with human febrile disease in Tanzania and has been isolated from *Culex rubinotus* and other species of mosquitoes, rodents, and sentinel hamsters in Kenya, South Africa, Zimbabwe, and Mozambique. The virus is pathogenic for infant and weaned mice by all routes of inoculation; it causes CPE in HeLa and primary hamster kidney cells and causes plaques in Vero and LLC-MK2 cells. It has been used as a model to study the pathogenesis of flaviviruses in mice (250).

Bussuquara Virus

Bussuquara virus was originally isolated from a sentinel monkey in Brazil in 1956 (158). The virus is quite distinct from other flaviviruses by neutralization test (66,106). Infant mice are susceptible to intracerebral and intraperitoneal infection, but older mice are only susceptible to intracerebral infection. Adult hamsters inoculated by all routes develop viremia and antibodies but no illness (263). Bussuquara virus replicates and produces plaques in a wide variety of cell cultures, including primary duck and chick embryo, BHK-21, Vero, LLC-MK2, MA-104, and MA-111. Histopathologic lesions resembling yellow fever were found in the sentinel monkey from which the original isolation was made, but subsequent experimental infection failed to reproduce these findings. The virus is known in Brazil and Colombia, where it has been isolated from various mosquito species, and in Panama, where it was recovered from the blood of a patient with fever, anorexia, and joint pains lasting 4 days (the only known human case) (528). Antibody prevalence rates of 12% have been found in humans in Panama. The virus has been repeatedly isolated from *Proechimys guyannensis* rodents in Brazil; experimentally inoculated *Proechimys* develop viremia. Mosquitoes of the genus *Culex* are the principal vectors. No control measures are applicable.

Edge Hill Virus

This flavivirus was isolated in 1961 from *Aedes vigilax* mosquitoes collected at Edge Hill, a suburb of Cairns, Queensland, Australia (119). On the basis of subsequent isolations and serosurveys, Edge Hill virus has a wide distribution in eastern Australia (120,206). By antigenic analysis, the virus was placed in the Uganda S virus complex (66). However, by nucleic acid hybridization, the virus was found genetically related to dengue type 2 virus (37). Edge Hill virus causes CPE and plaque formation in PS, BHK-21, and Vero cell cultures. The virus is pathogenic for newborn mice inoculated ip and ic and for weanling mice inoculated ic.

Human infections have been recognized by serological surveys in eastern Australia. A single case of human disease occurring in 1990 was attributed to Edge Hill virus on the basis of IgM serological responses (1). The patient,

a 64-year-old farmer from southeast Queensland, had a syndrome resembling epidemic polyarthritis (Ross River virus disease), with fever, myalgia, arthralgia, and fatigue. Symptoms recurred when medication (prednisone) was withdrawn, but finally resolved after 12 months. Sporadic cases must be distinguished from dengue fever (which cross-reacts serologically) and Ross River virus infection.

Edge Hill virus is transmitted between *Ae. vigilax* mosquitoes and mammals, principally wallabies and bandicoots. Virus isolates have been made also from *Culex annulirostris* and *Anopheles meraukensis*.

Ilheus Virus

Ilheus virus was first isolated from a pool of *Aedes* and *Psorophora* mosquitoes collected in Brazil in 1944. It was originally placed in the WN virus antigenic complex but was shown to be distinct from other members of the complex by neutralization test (66,106). The virus is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes but not for other laboratory animals. It produces plaques in primary rhesus kidney cells and various continuous cell lines (BHK-21, Vero, LLC-MK2, and PS) but not in avian cells. Pathologic features are described only in mice, which develop typical encephalitis. A total of eight human infections have been documented by virus isolation—five with mild febrile illness, headache, and myalgias; one with encephalitis; and two which were asymptomatic. These cases occurred in Brazil, Trinidad, Columbia, and Panama (263). The virus was inoculated into twenty cancer patients by Southam and Moore (525); three individuals developed CNS signs. Diagnosis is by virus isolation from blood or serologic tests, which are complicated by cross-reactions with other flaviviruses. The transmission cycle in nature involves wild birds and mosquitoes; at least eight genera of mosquitoes have yielded virus, but most isolations have been made from *Psorophora* spp. The potential link to the emergence of Rocio virus has been discussed above. No control measures are applicable.

Kokobera Virus

Kokobera virus was first isolated in 1960 from *Culex annulirostris* mosquitoes collected in northern Queensland. The virus is a member of the JE virus complex, and is thus related to the other Australian pathogens MVE and Kunjin viruses. Kokobera virus causes CPE and plaques in BHK-21, Vero, and PS cells and is pathogenic for infant and weanling mice by the ic route and for infant mice inoculated intraperitoneally. Serological surveys in New South Wales have demonstrated presumed human infections (206,208). Evidence for human disease was provided by serological analyses of three patients from this region in 1984 and 1985 (41). The disease was characterized by fever, fatigue, headache, neck pains, painful, stiff and (in one case) swollen joints, and in two of three cases, maculopapular, slightly pruritic, desquamating rash. In all three patients, there was a prolonged convalescence, with joint pains persisting for months.

The distribution of Kokobera virus includes eastern Australia and New Guinea. The virus is probably transmitted between *Aedes vigilax* and wallabies and kangaroos. However, virus isolations have also been made from *Culex annulirostris*, and the ecology of this virus remains poorly defined.

Koutango Virus

Koutango virus was isolated from a gerbil (*Tatera kempi*) in Senegal in 1968. Subsequent strains have been recovered from other rodent species (*Mastomys, Lemnyscomys*) and from several genera of ticks (*Rhipicephalus, Hyalomma, Alectorobius*) in Senegal and the Central African Republic. No naturally acquired human disease is reported, but a laboratory infection characterized by fever, headache, joint pains, and rash occurred in Senegal.

Kunjin Virus

Kunjin virus was first isolated from *Culex annulirostris* mosquitoes in Australia in 1960. It is a member of the JE virus antigenic complex and is thus antigenically closely related to MVE virus. The virus is pathogenic for infant mice by intracerebral and intraperitoneal routes and for weaned mice by intracerebral inoculation. It forms plaques in primary chick embryo, Vero, and PS cell cultures. Kunjin virus strains isolated in widely separated regions of Australia are genetically homogenous (138,331). The nucleotide sequence has a very high level of homology with respect to WN virus, implying an evolutionary relationship (92). Human disease has been reported from Australia. Two accidental laboratory infections were characterized by mild febrile illness—one with rash and one with lymphadenopathy (6). The first severe case with encephalitis mimicking MVE was described in 1986 (397). Of 45 cases diagnosed as MVE during the 1974 epidemic, 5 cases may in fact have been due to Kunjin virus infection. Cases of Kunjin encephalitis have been reported from Western Australia. Of 20 encephalitis cases diagnosed between 1978 and 1991, 2 (10%) were due to Kunjin and 18 were caused by MVE virus (338). The clinical disease resembles MVE, but is probably less severe. Serologic diagnosis is complicated by cross-reactions with MVE. Kunjin virus was isolated from the spinal cord of a horse with severe encephalomyelitis in New South Wales (16). *Culex annulirostris* is believed to be the principal vector, and wild birds and mammals are viremic hosts. The virus has also been isolated in Sarawak and Thailand.

Rio Bravo Virus

Rio Bravo virus was first isolated from a Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) in California in 1954. Subsequently, many other isolations have been made from bats in California, Texas, New Mexico, and Sonora, Mexico (263). The virus is pathogenic for infant mice. Experimentally inoculated monkeys and hamsters develop infection but no illness. A single naturally acquired human case with febrile illness was reported. Laboratory infection (538) resulted in moderately severe illness in three of five individuals who experienced aseptic meningitis, orchitis, or oöphoritis. The virus is not arthropod-borne and does not multiply in inoculated mosquitoes. It is probably transmitted bat to bat by direct contact or aerosol.

Sepik Virus

Sepik virus was isolated from *Mansonia septempunctata* mosquitoes in New Guinea in 1966. Several other isolates have been made from *Ficalbia* and *Armigeres* spp. mosquitoes in New Guinea. Antigenically, Sepik virus is a close relative of Wesselsbron virus. A single human case of febrile illness with headache, requiring hospitalization, has been recorded (263).

Spondweni Virus

Spondweni virus was originally isolated from *Mansonia uniformis* mosquitoes collected in Northern Zululand, South Africa, in 1955 (263). It is ungrouped but is most closely related antigenically to Zika virus. The virus is pathogenic for infant and weanling mice inoculated intracerebrally, produces CPE in primary hamster and monkey kidney cells, and produces plaques in Vero and LLC-MK2 cells. A virus isolated from the blood of an anicteric child with fever and headache and first thought to be Zika virus was later identified as Spondweni virus; however, recent analysis of this virus strain by monoclonal antibodies suggests that it is a mixture of yellow fever and another flavivirus most closely resembling Spondweni. A volunteer inoculated with this virus developed a mild febrile illness (26). During investigations in South Africa, two persons acquired laboratory infections characterized by fever, chills, aches and pains, nausea, and epistaxis (365). Wolfe et al. (597) reported a case in an expatriate living in Burkino Faso; symptoms were fever, dizziness, nausea, myalgia, headache, photophobia, and a maculopapular pruritic rash. Treatment is symptomatic. Diagnosis is by virus isolation from blood or serology. Spondweni virus has been isolated from at least seven mosquito species, but most isolates have come from *Aedes circumluteolus* in South Africa. Antibody has been found in cattle, sheep, and goats in South Africa, but the vertebrate hosts involved in transmission are unknown. No control measures are applicable.

Usutu Virus

Usutu virus was first isolated from *Culex naevei* mosquitoes in South Africa in 1959. Newborn and weaned mice are susceptible to fatal encephalitis by the intracerebral route. A case of human infection with fever and rash was reported from the Central African Republic in 1982. The virus has a wide distribution in sub-Saharan Africa. *Culex* mosquitoes and birds are responsible for transmission in nature. *Culex perfuscus* appears to be an important vector. Isolations of the virus have also been made from *Mansonia africana* and *Praomys* mice in the Central African Republic and from *Coquilletidia aurites* mosquitoes in Uganda.

Wesselsbron Virus

Wesselsbron virus was first isolated from a dead lamb during an epizootic in South Africa in 1955. It is antigenically closely related to Sepik virus and shows some cross-reactivity with yellow fever virus. Wesselsbron immune monkeys develop reduced viremias when challenged with yellow fever virus (228). The virus kills infant and weanling mice by all routes of inoculation. It produces CPE in BHK-21 cells and plaques in Vero and LLC-MK2 cells. Wesselsbron infection in sheep is of veterinary public health importance in southern Africa, where it causes abortion and death of newborn lambs and pregnant ewes (582). Some goat strains are highly susceptible to lethal infection. A mild, febrile disease occurs in cows. A syndrome of hydrops amnii in pregnant ewes, with prolonged gestation, maternal deaths, and fetal malformation (arthrogyphosis, hydranencephaly, hypoplasia, or segmental aplasia of the spinal cord, neurogenic muscular atrophy, and inferior brachygnathia), has been associated with both wild virus infection and use of a live attenuated vaccine (90). Pathologic changes in lambs include jaundice, focal necrosis of liver cells, Councilman-like bodies, and mild periportal inflammation (318). The clinical features, epidemiology, and pathologic changes in livestock resemble Rift Valley fever, from which it must be distinguished. Naturally acquired human cases of febrile illness occurred in South and West Africa, and there have been several reports of laboratory infections. Human illness is characterized by a short incubation period (2 to 4 days) and sudden onset of fever, chills, myalgia, hyperesthesia of the skin, hepatosplenomegaly, and maculopapular rash. In severely ill persons, signs of CNS involvement have been noted (363). There have been no fatalities, nor have signs of severe liver dysfunction similar to those seen in livestock been observed in humans. Clinical laboratory studies show leukopenia and elevated serum transaminase levels. Treatment is supportive. Diagnosis is by virus isolation from blood or by serology. The virus has been recovered from pharyngeal swabs. Serologic cross-reactions present difficulties in persons with prior flavivirus infection. The transmission cycle involves

mosquitoes. Species incriminated most frequently are *Aedes caballus-juppi*, *Aedes lineatopennis*, and *Aedes circumluteolus* in South Africa and numerous other *Aedes* spp. elsewhere in Africa. The virus is known to occur in Zimbabwe, Cameroon, Nigeria, Senegal, Ivory Coast, Central African Republic, Uganda, Kenya, Madagascar, and Thailand (263). The vertebrate hosts involved in transmission are uncertain; domestic livestock develop high viremias, as do experimentally infected gerbils.

No specific control measures are recommended for prevention of human infection. Special care is required when manipulating this virus in the laboratory. Because of the veterinary hazard, work with the virus in the U.S. is restricted by the Department of Agriculture.

Zika Virus

Zika virus was first isolated from a sentinel monkey in Uganda in 1947. The virus is not assigned to a subgroup but is most closely related to Spondweni, yellow fever, and Uganda S viruses. Zika immunity suppressed viremia in monkeys challenged with yellow fever (228). The virus kills infant and weanling mice by all routes of inoculation. It produces viremia but no illness in monkeys. Rabbits and guinea pigs inoculated by peripheral routes develop antibodies. Plaque assays may be performed in primary chick or duck embryo cell cultures. Pathologic changes in infected mice include encephalitis, myocarditis, and myositis. Serological surveys reveal a prevalence of human infection of up to 50% in many areas of Africa and in parts of Asia, but human disease has been rarely reported. Of approximately 14 diagnosed human cases, 1 was in a mosquito collector working in Senegal, 12 were naturally acquired cases that occurred in Senegal, Central African Republic (390), Nigeria (391) and Indonesia (417), and 2 were laboratory-acquired. Illness is characterized by fever, malaise, headache, and a maculopapular rash. Treatment is supportive. Diagnosis is by virus isolation from blood or serology; cross-reactions confuse interpretation, especially in persons with prior flavivirus exposures. The virus has been isolated from primatophilic *Aedes* spp. that also play a role in yellow fever transmission, e.g., *Aedes africanus*, *Aedes luteocephalus*, *Aedes vittatus*, *Aedes furcifer-taylori*, *Aedes metallicus*, and *Aedes opok*. Although the vertebrate hosts involved in transmission are not fully defined, it is likely that both nonhuman and human primates play a role and that the transmission cycle is similar to that of yellow fever. Multiple isolations have been made from *Cercopithecus* and *Erythrocebus* monkeys in West and East Africa. Explosive epizootics (without recognized disease) occur in monkey populations in East and West Africa. A single isolate has been recovered from a rodent (*Taterillus*) in Senegal, and isolations have been made from a large number of mosquitoes other than those listed above, raising the likelihood that the ecology of Zika virus is considerably more complex. No control measures are applicable.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH HEMORRHAGIC FEVER

Yellow Fever Virus

Historical aspects of yellow fever have been reviewed by Strode (536) and Monath (375). The disease was recognized as a clinical entity in 1648 in Yucatan. Tropical areas of the Americas were subject to large outbreaks in the 17th, 18th, 19th, and early 20th centuries, and the disease occurred in epidemic foci as far north as Boston and Halifax; it also appeared during the 18th century in Italy, France, Spain, and England. As late as 1905 there were 5,000 cases and 1,000 deaths in port cities of the southern U.S.

Mosquitoes were suggested as the vector of yellow fever by Nott in 1848, but this theory was not seriously proposed until 1881 by Carlos Finlay. In 1900, Walter Reed demonstrated a filterable agent in the blood of patients and showed transmission by *Aedes aegypti* mosquitoes. Despite suggestions to the contrary, yellow fever was thought to be transmitted exclusively between human beings by *Aedes aegypti*. Investigations by Soper (524) resulted in the concept of jungle yellow fever, later shown in both tropical America and East Africa to involve wild monkeys and sylvatic mosquito species.

In 1927, Mahaffy and Bauer first isolated the virus by inoculation of a rhesus monkey with the blood of a patient in Ghana; this was the source of the Asibi strain, parent of the 17D vaccine (533). In 1937, Theiler and Smith (546) reported attenuation of the Asibi strain by passage in chick embryo tissue and demonstrated use of the modified virus (17D) for human immunization.

Yellow fever has continued to be a major public health problem in the Americas. Cases are of the jungle type, and no *Aedes aegypti*-borne outbreaks have been reported in over 50 years. In Africa, however, large epidemics involving thousands of cases continue to occur, and the disease incidence has dramatically increased in recent years (12). Both *Aedes aegypti* and various sylvatic vectors have been responsible for epidemic transmission in Africa.

Infectious Agent

Yellow fever virus is the prototype of the flavivirus genus. It has been used as a model for elucidation of the flavivirus genome structure and replication strategy (see Chapter 30) and for studies on the molecular basis of antigenic structure/function and virulence.

Yellow fever is not placed within an antigenic subgroup or complex by plaque reduction neutralization tests (66,106); however, it is antigenically more closely related to Banzi, Wesselsbron, Bouboui, Zika, and Uganda S viruses than to other flaviviruses. By virtue of its relationship to Zika virus, Spondweni virus may be considered indirectly linked to yellow fever.

Cross-protection between yellow fever and many flaviviruses can be demonstrated using the sensitive intraperitoneal test in infant mice. In more epidemiologically meaningful tests of cross-protection, prior immunization with Wesselsbron, Zika, and dengue viruses caused a significant reduction in viremias of monkeys challenged with virulent yellow fever virus (228,545).

Antigenic differences have been shown between strains of yellow fever virus. By polyclonal antibody absorption techniques, strains from tropical America and Africa are distinct, as are 17D vaccine and parent Asibi viruses (88). Monoclonal antibodies have been described that are specific for yellow fever virus, for the 17D vaccine strain or for vaccine substrains 17D-204 and 17DD (22,501). Monoclonal analyses suggest that yellow fever virus is closer to mosquito-borne viruses than to tick-borne viruses. Some of these relationships (e.g., to Banzi, Uganda S, and Zika) fit previous concepts from polyclonal antibody analyses, but many others do not.

Monoclonal antibodies have not provided an antigenic classification of wild yellow fever strains by geographic origin, and further studies in this direction are required. In one limited analysis, Central and West African strains could be distinguished by immunoprecipitation of NS1 protein by anti-NS1 monoclonal antibodies (111). RNA fingerprinting and limited sequencing have delineated four yellow fever topotypes (see section entitled "Molecular Epidemiology," below). Strains from South America and Africa are also distinguishable by the electrophoretic migration and carbohydrate content of their E proteins.

Differences have been found between wild yellow fever virus strains in terms of their virulence for mice and monkeys and infectivity for mosquitoes (23), but some results are conflicting and reflect multiple variables, including mouse strain, virus passage level, and heterogeneity of virion subpopulations in virus stocks.

The molecular basis for yellow fever virulence has been partially elucidated by comparison between wild-type and vaccine viruses at the sequence level (187) and between vaccine strains with differing virulence patterns (254,255, 496). For a review, see section "Molecular Basis of Virulence," above.

Yellow fever virus can be propagated in a wide variety of primary and continuous cell cultures. Vaccine strains (17D and French neurotropic viruses) grow to higher titer and produce more evident CPE and plaques than do wild strains in various continuous monkey kidney (MA-104, Vero, LLC-MK2), rabbit kidney (MA-111), baby hamster kidney (BHK), and PS cell lines as well as in primary chick and duck embryo fibroblast monolayers. Wild yellow fever virus strains can also be propagated in these cell cultures, but plaque formation is inconsistent and variable from strain to strain. Viral growth can be detected by immunofluorescence in advance of the appearance of plaques. Both 17D vaccine and the parent Asibi virus grow in cell cultures of human origin (e.g., Chang liver cells, Henle embryonic intestine, HeLa, KB). Persistent infection of these cells has been described. The virulence properties of virus from carrier cultures can be altered; passage in HeLa cells, in particular, has been associated with a loss of viscerotropism for monkeys (24,124,212).

Mosquito cell cultures are useful for primary isolation and are more sensitive than Vero cells or infant mice. *Aedes pseudoscutellaris* (AP-61), cloned *Aedes aegypti*, and *Aedes albopictus* cells are susceptible; infection is generally assessed by immunofluorescence and/or subpassage to mice or Vero cells. AP-61 cells consistently show CPE. Intrathoracic inoculation of mosquitoes (*Toxorhynchites* or *Aedes aegypti*) is also useful for isolation or assay of yellow fever virus. After a 10- to 16-day incubation period, mosquitoes can be examined directly for virus by immunofluorescence or can be subpassaged to a susceptible host (e.g., suckling mice). Passage of some wild-type and vaccine strains in C6/36 cells resulted in the appearance of temperature-sensitive virus.

In vertebrate species, yellow fever virus produces both neurotropic and viscerotropic patterns of infection. Viscerotropism reflects the pathogenicity of yellow fever virus for human or nonhuman primates infected by the peripheral route; disease is principally characterized by hepatic pathology. Rhesus and cynomolgus macaques, as well as certain neotropical monkeys, are highly susceptible. Monkeys intracerebrally inoculated with wild-type virus develop encephalitis but die of viscerotropic yellow fever.

The European hedgehog (*Erinaceus europaeus*) is the only nonprimate experimental animal that develops severe visceral lesions in response to yellow fever infection. Hamsters inoculated intraperitoneally with some virus strains (e.g., Asibi) are partially susceptible (scattered deaths), but they resist lethal infection with other virus strains. Rabbits resist intracerebral and peripheral challenge but form antibodies. Newly hatched chickens develop viremic infection without disease.

The neurotropic properties of yellow fever virus are most apparent in mice; infant mice are highly susceptible to encephalitis after intraperitoneal or intracerebral inoculation. Considerable variation in neuropathogenicity for mice exists between virus strains (23). Older mice are susceptible by the intracerebral route, as are guinea pigs.

Pathogenesis and Pathology

Neurotropic yellow fever infection of mice has been used as a model system for studies on the pathogenesis of flavivirus encephalitis (see general Pathogenesis and Pathology, above). The discussion to follow deals solely with the viscerotropic disease in humans and nonhuman primate models.

The typical yellow fever lesion is marked by cloudy swelling, then by coagulative necrosis of hepatocytes in the midzone of the liver lobule, sparing cells bordering the

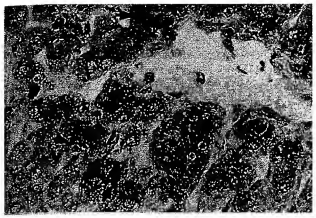


FIG. 17. Postmortem liver sample from a patient with yellow fever, showing midzonal necrosis and hemorrhage, as well as sparing of cells bordering the central vein. Yellow fever viral antigen (*arrows*) is demonstrated by immunohistochemistry (alkaline phosphatase antialkaline phosphatase technique) and is present both in cells undergoing necrotic change and in the cells preserved around the central vein. ×500.

central vein (Fig. 17). Eosinophilic degeneration of hepatocytes results in the formation of Councilman bodies and intranuclear eosinophilic granular inclusions (Torres bodies). On an ultrastructural level, Councilman and Torres bodies consist of amorphous material without any viral structures. Multi- and microvacuolar fatty change is nearly always present, especially after the eighth day of illness. An inflammatory response is absent or mild. The reticulin framework is preserved, and healing is complete in recovered cases. Typical changes have been seen in biopsy specimens taken as early as the third day of illness; interpretation of biopsy or necropsy material obtained after the tenth day is often difficult. In cases in which death is delayed, partially disintegrated Councilman bodies consisting of small, irregular, granular, ochre-colored bodies ("Villela bodies") may be found, especially in the midzone.

Renal glomerular changes are relatively insignificant compared to acute tubular necrosis and fatty metamorphosis, which may be marked. Schiff-positive transformation of the glomerular basement membrane has been described and has been linked to altered permeability to proteins and albuminuria (378). The myocardial fibers show cloudy swelling, degeneration, and fatty infiltration. The brain may show edema and petechial hemorrhages. Lymphocytic elements in the spleen and lymph nodes are depleted, and large mononuclear or histiocytic cells accumulate in the splenic follicles.

Our present understanding of yellow fever pathogenesis and pathophysiology relies extensively on experimental observations in rhesus monkeys (380,548). After inoculation, the virus replicates in regional lymph nodes and then spreads to other tissues, including liver, spleen, bone marrow, and cardiac and skeletal muscle. The hepatic parenchyma is the principal target organ, and hepatocellular damage is undoubtedly mediated directly by viral infection. Serial examination of liver tissue from infected rhesus monkeys reveals the earliest cytopathologic changes and appearance of immunofluorescent viral antigen in Kupffer cells (548). Lymphocytic necrosis in the germinal centers of the splenic periarteriolar lymphocytic sheath and necrosis of germinal centers in lymph node follicles are prominent findings in the rhesus monkey (380). Yellow fever (17D) virus replicates to high titer in human peripheral monocyte cultures and in peripheral blood lymphocytes stimulated by phytohemagglutinin (588).

The pathogenesis of the renal lesion is uncertain. In the experimentally infected rhesus monkey, changes in renal function are most revealing during the interval between 36 and 12 hr before death (380). Oliguria was interpreted to reflect intrarenal changes in blood flow secondary to decreased effective blood volume, whereas the development of acute tubular necrosis very late in the infection was the result of the generalized circulatory collapse. Acid-base disturbances, as well as changes in the distribution of water and electrolytes, in cardiac muscle and brain have been described and may be important pathophysiological events.

The pathogenesis of the bleeding diathesis in yellow fever is also complex. Decreased synthesis of vitamin K-dependent coagulation factors by the diseased liver is an important part of the hemorrhagic disorder, but disseminated intravascular coagulation and altered platelet function may play a role in severe and fatal cases.

Genetic factors may be important in determining individual host responses to yellow fever infection but have not been well defined. Older reports emphasize the severity of the disease in whites compared to blacks, but it is not possible to separate genetic from acquired resistance factors (e.g., immunity to yellow fever or heterologous flaviviruses). Descendants of Dutch colonists in Surinam who survived typhoid and yellow fever epidemics had gene frequencies that were significantly different from those of a large Dutch control group, possibly indicating selection through genetic control of survival (114).

Clinical Features

The incubation period is usually 3 to 6 days. The clinical spectrum varies from very mild, nonspecific, febrile illness to a fulminating, sometimes fatal disease with pathognomonic features (378,536).

Severe yellow fever begins abruptly with fever, chills, severe headache, lumbosacral pain, generalized myalgia, anorexia, nausea and vomiting, and minor gingival hemorrhages or epistaxes. Despite a persistent or rising temperature, the pulse may decrease (Faget's sign). This syndrome, lasting approximately 3 days, corresponds to the *period of infection*, during which yellow fever virus is pre-

sent in the blood. It may be followed by a *period of remission*, with defervescence and mitigation of symptoms, usually lasting up to 24 hr. Fever and symptoms reappear with more frequent vomiting, epigastric pain, prostration, and the appearance of jaundice (*period of intoxication*). Viremia is generally absent, and antibodies appear during this phase. The bleeding diathesis is manifested by coffee-grounds hematemesis (vomito negro), melena, metrorrhagia, petechiae, ecchymoses, and diffuse oozing from the mucous membranes. Dehydration results from vomiting and increased insensible losses. Renal dysfunction is marked by a sudden increase in albuminuria and diminishing urine output. Death (in 20% to 50% of severe yellow fever cases) occurs usually on the seventh to tenth day of illness and is preceded by deepening jaundice, hemorrhages, rising pulse, hypotension, oliguria, and azotemia. Hypothermia, agitated delirium, intractable hiccups, hypoglycemia, stupor, and coma are terminal signs. Leukopenia occurs during the acute phase of illness. Other laboratory abnormalities include elevation of bilirubin and serum transaminases, thrombocytopenia, prolonged clotting and prothrombin times, and S-T wave changes in the electrocardiogram.

Convalescence is sometimes prolonged, with profound asthenia lasting 1 to 2 weeks. Late death, occurring at the end of convalescence or even weeks after complete recovery from the acute illness, is a rare phenomenon attributed to cardiac complications or renal failure. The duration of icterus in surviving cases is unknown. Elevations of serum transaminase levels have been documented to persist for at least 2 months after onset of yellow fever.

Diagnosis

Mild yellow fever cannot be distinguished clinically from a wide array of other infections. In the presence of jaundice and the other signs of severe yellow fever, conditions that must be differentiated include viral hepatitis, falciparum malaria, leptospiral infections, Rift Valley fever, typhoid, Q fever, typhus, and surgical, drug-induced, and toxic causes. The other viral hemorrhagic fevers, which usually present without jaundice, include dengue, Lassa, Marburg, and Ebola virus diseases; Bolivian and Argentine hemorrhagic fevers; and Congo/Crimean hemorrhagic fever.

Specific diagnosis depends on histopathologic study, isolation of the virus, or demonstration of viral antigen or a specific antibody response. The virus is most readily isolated from serum obtained during the first 4 days of illness, but it may be recovered from serum up to the fourteenth day and, occasionally, from liver tissue at death. Isolation attempts from clinical specimens can be made by intracerebral inoculation of mice, intrathoracic inoculation of *Toxorhynchites* mosquitoes, or inoculation of mosquito cell cultures. The *Aedes pseudoscutellaris* cell line has the advantage of high sensitivity and a relatively short incubation time (3 to 6 days) to detection of virus (by immunofluorescence using polyclonal or monoclonal reagents). Polymerase chain reaction or nucleic acid hybridization may find application for early detection of virus in such samples (443). Viral antigen or IgM-antigen complexes in serum may be detected by immunoassay (384), affording a rapid, early diagnosis. A comparison of various techniques showed that the antigen-capture ELISA has a sensitivity of approximately 70%, and it may detect noninfectious antigen in poorly handled specimens. PCR has shown promise for rapid detection of viral genome in serum. A definitive postmortem diagnosis may be made by detection of yellow fever antigen in liver tissue sections by immunocytochemical staining (389). It should be emphasized that in living patients, liver biopsy is contraindicated as a diagnostic procedure due to the high risk of hemorrhage.

Serologic methods useful in the diagnosis of yellow fever include the usual HI, CF, and neutralization tests, single radial hemolysis, indirect immunofluorescence, ELISA, and RIA. The HI, IFA, and neutralization antibodies appear within a week of onset; CF antibodies appear later. The plaque reduction neutralization test has now largely replaced the less sensitive test in mice. Paired acute- and convalescent-phase specimens are required to establish the diagnosis by the rise in antibody titer. Cross-reactions complicate serodiagnosis in cases with prior exposure to heterologous flaviviruses.

Antigens suitable for serological assays are prepared from infected mouse brain tissue, cell culture fluids, or extracted mosquito cell culture membranes. Determination of IgM antibodies by the indirect fluorescent antibody technique or ELISA may indicate recent infection. The duration of IgM antibodies is uncertain, however, and appears to be quite variable. In persons vaccinated with 17D virus, detectable IgM neutralizing antibodies are present as long as 18 months after immunization. IgM antibodies show relative specificity, but cross-reactions in ELISA are sometimes found in patients with prior flavivirus exposures.

The use of yellow fever 17D vaccine may confound serodiagnosis. In persons without prior flavivirus exposure, the vaccine induces a neutralization test seroconversion with a low titer (1:10 to 1:40) of HI antibodies and no detectable IFA or CF antibodies. However, in persons with preexisting flavivirus antibodies, vaccination may result in marked rises in yellow fever and heterologous HI and CF antibodies. The patterns produced may be broad or heterotypic; in some cases, however, homotypic responses are seen (a specific rise in yellow fever HI or CF antibodies), similar to those seen in recent natural infection.

Treatment

Treatment is supportive. Most patients with yellow fever have not benefitted from the availability of modern intensive care, and it is unknown to what extent fluid manage-

ment and correction of hypotension and electrolyte and acid-base disturbances would reverse the apparently inexorable course of severe yellow fever. A number of compounds with antiviral activity *in vitro* have been described, including ribavirin and derivative compounds [for review, see (378)]. Ribavirin suppresses yellow fever replication *in vitro*, but at concentrations higher than those achievable *in vivo*. Trials of ribavirin in experimentally infected monkeys showed no therapeutic effect. Gamma-interferon treatment of monkeys resulted in delayed onset of viremia and illness but had no effect on survival.

Epidemiology

Yellow fever is a zoonotic disease. The primary transmission cycle involves wild nonhuman primates and various sylvatic (tree-hole-breeding) aedine mosquitoes. Humans may be tangentially exposed when they encroach on this cycle (so-called jungle yellow fever), and epidemic spread from human to human can subsequently be continued by sylvatic vectors (Fig. 15). Alternatively, the domestic mosquito, *Aedes aegypti*, which lives in close relationship with humans, may transmit the virus, with humans being the sole viremic hosts in the cycle (*Aedes aegypti*-borne yellow fever or "urban yellow fever"). Detailed reviews of the ecology of yellow fever are available (94,153,374).

Yellow fever occurs throughout much of tropical South America and sub-Saharan Africa. Within this region, viral activity may be intermittent and quite localized. The distribution of reported cases gives only a partial picture of the natural circulation of yellow fever virus and gives a misleading estimate of risk to travelers (Fig. 19). The virus is potentially present throughout the tropical rainforest and bordering grassland areas of South America and Africa.

The annual incidence of officially reported yellow fever cases is 50 to 300 cases in tropical America and up to 5,000 cases in Africa (Fig. 18). These data represent a significant underestimate of the true morbidity, as shown by investigations of various epidemics. The incidence in Africa has increased dramatically in recent years, principally due to a series of annual outbreaks in Nigeria between 1986 and 1991 that probably involved hundreds of thousands of cases [Fig. 19 and (375)]. The virus spread from Nigeria to Cameroon in 1990, causing an epidemic that may have involved up to 20,000 cases and 1,000 deaths (570). The most recent outbreak in West Africa occurred in Ghana (1993). In East Africa, the first yellow fever epidemic in over 25 years occurred in 1992 to 1993 in western Kenya.

In tropical America the incidence of jungle yellow fever is highest during months with peak rainfall, humidity, and temperature (January to March). In Africa, transmission by *Aedes aegypti* and tree-hole-breeding mosquitoes peaks during the late rainy season and early dry season. In tropical America, jungle yellow fever principally affects young adult males. The age/sex distribution reflects the higher incidence of exposure to *Haemagogus* vectors during woodcutting and forest-clearing activities in the forest. In Africa, background immunity (natural and vaccine-induced) is the principal factor determining the age distribution of cases. In outbreaks affecting immunologically virgin populations (e.g., in Ethiopia, 1960 to 1962), all ages are equally affected. In West Africa, a high level of acquired immunity in adults has resulted in high attack rates in children. A slight excess of cases in males is typical of African epidemics, and is probably due to increased exposure to sylvatic vectors during the period around sunset. The ratio of infections to clinical cases ranges between 2:1 to 20:1. Prior infection with one or more heterologous flaviviruses appears to mitigate severity and increase the infection:case ratio (381). The case-fatality ratio has varied widely in different epidemics, possibly reflecting virus strain variation in virulence.

Yellow fever has never occurred in Asia. Possible explanations include (a) cross-protection afforded by dengue immunity and (b) low vector competence of Asian strains of *Aedes aegypti*; experimental evidence in support of both hypotheses has been presented [for review, see (374)].

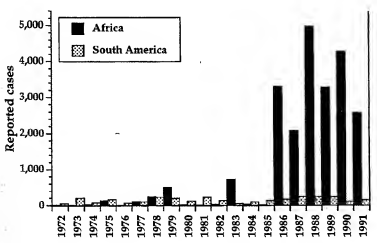


FIG. 18. Officially-reported cases of yellow fever in South America and Africa, 1972–1991, showing the increase in incidence in Africa in recent years, due to a series of epidemic in West Africa (principally Nigeria), and (in part) to improved reporting.

FIG. 19. Yellow fever: officially reported cases to the World Health Organization during the decade 1982–1991.

Ecology of Yellow Fever in Tropical America

Howler monkeys (*Alouatta* sp.), spider monkeys (*Ateles* sp.), squirrel monkeys (*Saimiri* sp.), and owl monkeys (*Aotus* sp.) are effective viremic hosts and commonly develop fatal infections, whereas capuchin monkeys (*Cebus* sp.) and wooly monkeys (*Lagothrix* sp.) are susceptible to viremic infection but usually do not develop clinical signs. This unstable host-parasite relationship may reflect the relatively recent introduction of the virus, possibly at the time navigation was established between Africa and America during the 15th century. Other South American vertebrates, including edentates, marsupials, and rodents, are now believed to play a negligible role in the yellow fever transmission cycle, although further study appears warranted.

Mosquitoes of the genus *Haemagogus* are the principal vectors of jungle yellow fever in tropical America. *Haemagogus* breed in tree holes and feed in the forest canopy during the midday hours, but they also have been found biting humans in forest clearings and even inside houses in villages near the forest. Transovarial transmission of yellow fever virus in *Haemagogus* has been experimentally demonstrated. This phenomenon may explain, in part, maintenance of the virus during prolonged dry seasons, when adult vector populations are diminished. The relatively drought-resistant mosquito *Sabethes chloropterus*, a relatively inefficient vector, may also play a role in virus survival.

The development of anti-*aegypti* campaigns in Latin America during the 20th century culminated in eradication of the vector from most countries surrounding the Amazon Basin and the disappearance of urban yellow fever after 1942. However, within the last 15 years, *Aedes aegypti* has reinvaded many areas [see "Dengue Fever" and (176,376)]. The urban vector again exists in or near areas of enzootic yellow fever, raising the specter of future urban outbreaks.

Ecology of Yellow Fever in Africa

All species of cercopithecid and colobid monkeys, and some prosimians tested have proved to be effective viremic hosts, circulating virus for several days or more at sufficient titers to infect mosquitoes (374,468). Infection infrequently results in illness or death, indicating a balanced host-parasite relationship.

Aedes africanus is responsible for year-round virus transmission in the humid equatorial African forests (Fig. 15). The ecologic zones bordering equatorial forest have assumed great importance in yellow fever ecology. Appropriately named the *zone of emergence* by Germain et al. (153), the savannah vegetational zones support large and concentrated populations of monkeys and vector mosquitoes. Viral activity intensifies during the rainy season and wanes during the dry season, when vector populations vir-

tually disappear. The principal species involved in sylvatic transmission and transmission to humans are *Aedes furcifer, Aedes africanus*, and *Aedes luteocephalus*. These species are responsible for interhuman spread during epidemics. Depending on location, other vectors that play a role in yellow fever transmission cycles include *Aedes vittatus, Aedes metallicus, Aedes opok, Aedes neoafricanus,* and *Aedes keniensis*. Surveillance of virus activity in sylvatic vectors collected in eastern Senegal over a 17-year period (1976 to 1993) has shown an impressive correlation with epidemics occurring throughout the West African region (12). The ecological events underlying the periodicity of virus amplification are unknown, but may reflect regional fluctuations in rainfall patterns (376).

In areas subject to extreme drying (e.g., in the dry northern Sudan and Sahel savannah zones of West Africa), yellow fever occurs in intermittent epidemic form, and human immunity patterns indicate little or no infection during interepidemic periods. In these areas, domestic water storage is intensively practiced, domestic *Aedes aegypti* populations are high, and introduction of yellow fever virus may result in explosive outbreaks. Urban areas along the West African coast are also susceptible. A large urban epidemic occurred in western Nigeria in 1987.

Vertical transmission of yellow fever virus has been documentcd experimentally in *Aedes aegypti* (3). Evidence for vertical transmission in nature has been obtained by virus isolation from male *Aedes furcifer* in West Africa. This mechanism ensures virus survival over the long dry season. Yellow fever virus has been isolated from *Amblyomma variegatum* ticks in the Central African Republic (494), raising the possibility that alternate vectors may play a role in dispersal or dry-season maintenance of the virus.

Yellow fever virus has been rarely isolated from other arthropods, including *Aedes dentatus, Coquilletidia fuscopennata*, and phlebotomine flies. Virus has been isolated from a bat in Ethiopia. These observations are of interest but probably bear little relationship to the ecology of yellow fever.

Molecular Epidemiology

RNA oligonucleotide fingerprinting and nucleotide sequencing have distinguished at least three geographic topotypes [one in South America and two in Africa (108,317)]. In Africa, nucleotide sequencing of the E protein gene distinguishes strains isolated in West Africa (E-genotype IA) and America (E-genotype IB) from those in Central and East Africa (E-genotype II), the east-west dividing line falling at a longitude separating Nigeria/Cameroon and the Central African Republic. The yellow fever gene pool representing all isolates appears to be very stable. Genetic drift is observed within each genotype, indicating a random mutation rate of 2.2 bases per year in the envelope gene. All of the African yellow fever virus genotypes are capable of rapid amplification, epidemic spread, and induction of highly lethal human infections, and all have demonstrated the capacity to cross from sylvan to urban (*Aedes aegypti*-borne) transmission cycles.

Prevention and Control

Vaccination

Yellow fever 17D is a safe, effective live viral vaccine prepared from infected chicken embryos under standards developed by the World Health Organization (466,521). Demonstrable immunity occurs in over 95% of vaccinees within 10 days. For the purposes of international certification, immunization is valid for 10 years, but various studies have shown persistence of antibodies for as long as 30 to 35 years; immunity is probably lifelong (437). Many countries in South America conduct immunization campaigns, and a high vaccine coverage in enzootic areas has limited the incidence of human disease. Vaccination coverage in African countries is highly variable (466). In 1988, a strong recommendation was made to include 17D vaccine in the Expanded Program on Immunization. In 1992, coverage in 11 African countries ranged between 20% and 80%. Cost-effectiveness of routine preventive immunization of infants compares favorably to emergency mass immunization for epidemic control (377).

Serious adverse reactions to 17D vaccine are extremely uncommon. No abnormalities in liver function tests are associated with 17D vaccination. Fewer than 10% of vaccinees experience headache and malaise. Allergic reactions occur at a very low rate (approximately 1 in 1 million), predominantly in persons with an allergy to eggs. The vaccine should not be given to individuals with immunodeficiency states (including clinically overt HIV infection) or on immunosuppressive drugs.

Neurological accidents are extremely uncommon and 14 of 20 such cases were in infants <6 months of age (369, 377); vaccine is now contraindicated for infants below this age. Encephalitis following yellow fever vaccine is due to virus neuoinvasion and direct viral injury; in all but one fatal case (10), the encephalitis resolved without sequelae.

The safety of yellow fever vaccination during pregnancy is a subject for debate. Taking the results of two studies into consideration, the risk of congenital infection (assessed by IgM antibody present in cord blood) approximates 1% to 2%, and was not associated with fetal abnormality or illness (400,559). The stage of pregnancy during which vaccine was administered was uncertain in many cases, and further studies are warranted. Given the susceptibility of the immature nervous system to 17D virus, as noted by the occurrence of postvaccinal encephalitis in infants, and the fact that transplacental infection occurs in a small number of cases, immunization during pregnancy should be avoided. This recommendation must, however, take into account the relative risk of natural infection, which may be as high as 20% to 30% during epidemics.

Vaccination results in a low-level viremia lasting 1 to 2 days and beginning 3 to 4 days after inoculation. The low magnitude of viremia and the fact that *Aedes aegypti* is refractory to oral infection with 17D virus preclude the possibility of natural transmission (and possible reversion) of vaccine virus.

Factors that may affect seroconversion to the vaccine include: (a) nutritional state; (b) simultaneous administration of other vaccines; (c) preexisting heterologous flavivirus immunity; and (d) pregnancy. Children with kwashiorkor show marked impairment in antibody production after 17D vaccination. Persons administered 17D yellow fever and cholera vaccines simultaneously or 1 to 3 weeks apart showed reduced antibody responses to both vaccines (150). Other vaccine combinations and immune serum globulin can be used without interference. Studies with 17D vaccine produced in mouse brain and administered by scarification have shown a reduction in vaccine seroconversion in African population groups with prevaccination heterologous flavivirus antibodies. In persons given 17D chick embryo vaccine by the subcutaneous route, however, preexisting heterologous immunity did not interfere with the immune response. In one study (400) only 38% of pregnant women seroconverted, compared to 82% to 90% of nonpregnant controls. There are no data on the safety or efficacy of yellow fever vaccination of HIV-infected persons. Present recommendations are for vaccination of infected individuals without signs/laboratory parameters indicating immunosupression.

At the present time, vaccines produced by some of the world's 12 manufacturing institutes are contaminated with avian leukosis virus. Although undesirable, this contaminant has not been associated with the development of leukemia, lymphoma, or other cancers (576).

The French neurotropic vaccine, produced from infected suckling mouse brains, is no longer manufactured. The vaccine had the advantage of high stability and ease of administration (by scarification or multiple puncture). However, approximately 20% of vaccinees developed systemic symptoms, 3% to 4% developed meningeal signs, and 0.5% to 1.3% developed postvaccinal encephalitis. Neurologic accidents were more frequent in children than in adults; fatalities and permanent neurologic sequelae have been reported.

Other Preventive Measures

Areas infested with the domestic form of *Aedes aegypti* are at risk of the introduction and urbanization of yellow fever. Elimination of breeding sites (tires, artificial containers, etc.), treatment of potable water with temephos (Abate), perifocal spraying with organophosphorus insecticides, and use of Gambusia minnows are effective if applied in a well-administered and continually supported program.

In the case of an outbreak of *Aedes aegypti*-borne yellow fever, ground or aerial ULV application of adulticides may be used. The control of yellow fever epidemics involving wild vector species is more difficult, and little experience with vector control has been accumulated.

Aerial ULV applications of malathion were shown to be effective for the control of the yellow fever vector *Aedes bromeliae* breeding in false banana (*Musa ensetta*) plantations in Ethiopia. Ground and aerial applications of malathion rapidly suppressed populations of *Aedes africanus* in forest habitats in West Africa for a period of time believed sufficient to interrupt virus transmission. Aerial ULV was also used for the control of *Haemagogus* vectors in forested areas in eastern Panama in 1974.

Dengue Viruses (Dengue Hemorrhagic Fever)

Cases of hemorrhage and death had been described during outbreaks of classic dengue fever in Australia (in 1897), Greece (1928), and Formosa (1931) (258). In 1954 a febrile disease with hemorrhagic signs (Philippine hemorrhagic fever) occurred in epidemic form in Manila and was shown to be caused by dengue types 3 and 4. Dengue hemorrhagic fever was subsequently described in many other areas of Southeast Asia (197). The disease was first reported in China in 1985. The incidence of DHF in Asia has risen steeply in the last 20 years, and over 450,000 cases are now reported annually, making it a leading cause of morbidity. Case-fatality rates currently range from 1% to 10%, depending on the availability and sophistication of hospital care. Sporadic cases of DHF were recognized in the American region during the 1970s, and epidemic DHF appeared in the 1980s. The pathogenesis of DHF remains incompletely understood. Halstead proposed that the severe form of the disease had an immunopathologic basis and occurred in individuals previously sensitized by infection with a heterologous dengue serotype (190,192).

Infectious Agents

All four dengue virus serotypes cause DHF and DSS. In Thailand, dengue type 2 was the predominant virus associated with these syndromes until the 1980s, when dengue types 3 and 4 emerged as important serotypes. In Indonesia, dengue type 3 was most frequently isolated and associated with fatal cases between 1976 and 1978. In Cuba, Venezuela, and Brazil, DHF epidemics occurred when a specific strain of dengue type 2 was introduced 3 to 4 years after an outbreak of dengue type 1. The sequence of infecting serotypes, the interval between infections, and strain differences in virulence may be important determinants of the clinical and epidemiological patterns of DHF (62,175,190,191,493). Since an animal model of DHF has not been found, this question has eluded study. Two studies have indicated that viral replication in human mononu-

clear cells may provide a correlate of virulence (272,392). Comparison of strains associated with different disease outcomes at the genome level have also provided clues that virus-specified factors may contribute to the pathogenesis of DHF (388,463).

Pathogenesis and Pathology

The development of a satisfactory animal model of DHF has been an elusive goal. Unsuccessful attempts to develop models have included the sequential infection of nonhuman primates and of SCID mice reconstituted with human cells. The human disease is characterized principally by diffuse capillary leakage of plasma and a hemorrhagic diathesis. Increased vascular permeability results in hemoconcentration, decreased effective blood volume, tissue hypoxia, lactic acidosis, and shock. These pathophysiological events are probably mediated by cytokines, and pathologic findings in fatal human cases consequently reveal little evidence of direct viral injury (31,65,459). Gross pathological findings include: petechiae, ecchymoses, and focal visceral hemorrhages; serous and bloody effusions; retroperitoneal edema; and, in some cases, hepatic enlargement. On histopathologic examination, there are diffuse small-vessel changes in viscera and soft tissues with leakage of plasma (perivascular edema) and erythrocytes. The spleen and lymph nodes show proliferation of immature and mature lymphoid cells and plasmacytoid elements. Necrosis of thymus-depcndent areas of the spleen may be prominent. Hepatic lesions similar to other hemorrhagic fevers, though generally less severe, consist of central or paracentral focal necrosis, sinusoidal acidophilic (Councilman) bodies, hypertrophy of Kupffer cells, variable and mild fatty change, and patchy portal mononuclear cell infiltration. Bone marrow changes include maturational arrest of megakaryocytes. Focal dengue viral antigen has been demonstrated in skin, liver, and mononuclear leukocytes.

Prospective studies in Thailand have shown that over 90% of cases of DHF occur in persons who have previously been infected with at least one heterologous dengue serotype (62,192,493). Children with secondary immune responses were at significantly higher risk of developing DHF than those experiencing their first infection. Approximately 3% of persons who sustain secondary dengue infections develop DHF, whereas <0.2% of those with primary infections develop the syndrome. In infants who develop DHF as a consequence of primary dengue infection, the disease occurs mainly between 6 and 12 months of age in the presence of waning, passively acquired maternal antibody. The concept of antibody-dependent immune enhancement of dengue viral replication in monocytes has been proposed (190–193) to explain these observations (see Antibody-Dependent Enhancement, above). Dengue viruses were shown to replicate to higher titer in human mononuclear cells in the presence of cross-reactive nonneutralizing dengue antibodies than in the absence of antibodies. Infectious complexes of virions and IgG antibody gained access to monocytic cells via their Fcγ receptors. These findings led to the concept that the presence of enhancing antibodies increased the number of dengue-infected cells and that lysis or immune clearance of these cells led to the release of vasoactive mediators and procoagulants. Attempts to model this phenomenon *in vivo* by studying viremia levels in immunologically sensitized nonhuman primates provided equivocal results (199,479). However, recent evidence for the occurrence of ADE *in vivo* has been obtained from studies on the presence of NS1 antigen in the sera of patients with primary and secondary dengue infections (P.R. Young, personal communication, 1994). Acute phase sera from patients with secondary, but not primary, dengue infections were found to contain high levels of NS1 antigen, indicating enhanced numbers of infected cells in secondary infections.

Recent studies have focused on the role of T-cell activation and cell-mediated immunological clearance of dengue-infected monocytes in the pathogenesis of DHF. Both $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells have been detected in persons naturally infected with dengue viruses or immunized with live, attenuated vaccines (303,304). Cytotoxic $CD4^+CD8^+$ T-cell clones established from immune donors have demonstrated dengue serotype-specific, dengue serotype–cross-reactive, and flavivirus cross-reactive specificities. The $CD4^+$ clones lyse autologous dengue virus-infected cells in an HLA class II-restricted fashion; proliferate; and produce γ-interferon, IL-2, and GM-CSF when stimulated with dengue virus antigens (303,418). Memory $CD4^+CD8^-$ cell clones with HLA class I-restricted cytotoxicity also show dengue serotype cross-reactivity (57). The NS3, NS1, and E proteins appear to contain important epitopes for recognition by both $CD4^+$ and $CD8^+$ T cells (299).

A unifying hypothesis linking antibody-dependent enhancement of virus replication and T-cell activation has been proposed by Halstead (191) and Kurane and Ennis (305) (Fig. 20). In this model, the host has been previously infected with a dengue serotype and has cross-reactive serum antibodies and memory T cells. Upon infection with a heterologous dengue serotype, the formation of virus-antibody complexes leads to complement activation and enhanced infection of Fc γ receptor-bearing monocytic cells. Memory $CD4^+$ T lymphocytes activated by dengue antigens proliferate and produce γ-interferon, which in turn up-regulates both the expression of Fc γ R on monocytic cells (augmenting the infection of these cells by immune complexes) and the expression of HLA class I and II molecules involved in recognition of these cells by Tc cells. The dengue-infected monocytic cells are targets for $CD4^+$ and $CD8^+$ Tc cells, resulting in the release of cytokines or cell lysis and release of intracellular enzymes and activators. Evidence for enhanced $CD4^+$ and $CD8^+$ T-cell activation in DHF patients was provided by the finding of higher levels of soluble IL2 receptor, soluble CD4, and soluble

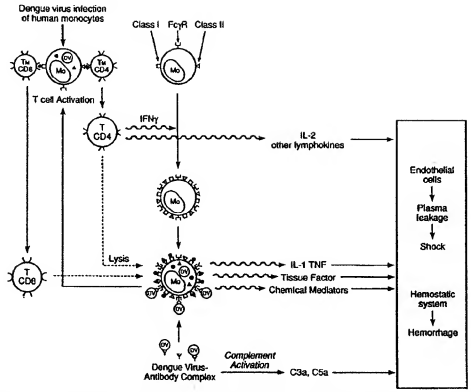


FIG. 20. Immunopathological mechanisms underlying the pathogenesis of dengue hemorrhagic fever, from Kurane and Ennis (305), with permission.

CD8 in sera of DHF than in dengue fever patients (301). However, the precise role of cytokines and tissue factor mediators of capillary leakage, shock, and hemorrhage in DHF remains a subject of research. Although several lines of evidence suggest that T-cell activation and release of mediators are key to the pathophysiology of DHF, it has proved difficult to demonstrate clear correlations with disease severity. Elevated levels of IL2 have been found in children with both dengue fever and DHF (301). IL2 is known to induce capillary leakage and complement activation when administered to humans. Elevated levels of TNF, IL-6, C3a and C5a, and histamine have been described in DHF (232,572). In children with DHF, levels of TNF-α and IL-6 were highest on the day of appearance of shock. TNF-α is released from activated monocyte/macrophages, is implicated in septic shock induced by bacterial endotoxins, and is thus a potentially important mediator in DHF/DSS. Bradykinin has not been implicated (126) as a mediator in DHF. High interferon-α levels are sustained for a week or more after onset (302), but the role of this molecule in immunoregulation/pathogenesis, clinical symptomatology, and control of dengue infection in the host remains to be defined.

The role of cytokine mediators in DHF has been investigated in mice by Chaturvedi and colleagues. These investigators described a unique cytokine called cytotoxic factor produced by splenic T cells. When administered to uninfected mice, cytotoxic factor induced vascular and blood-brain barrier permeability and lymphoid cell depletion, effects mimicking elements of DHF pathophysiology (82,115). Immunization of mice with cytotoxic factor prevented these effects.

The etiology of the hemorrhagic disturbance in DHF appears to be complex. Microvascular injury, thrombocytopenia, platelet dysfunction, and disseminated intravascular coagulopathy have been variously implicated [for review, see (191)]. Liver injury of significant degree may impair synthesis of vitamin K-dependent coagulation factors. Increased platelet turnover has been attributed to the direct attachment of dengue virus to platelets and to the presence of antiplatelet antibodies, with subsequent immune elimination. At a cellular level, it is probable that procoagulants are released from infected monocytic cells or endothelial cells activated by IL2 or other cytokines. In one study, dengue-infected monocytes were found to contain significantly higher levels of plasminogen activator-inhibitor, suggesting a mechanism for interference with the fibrinolytic system (283). A possible link between the immune response to dengue infection and the hemorrhagic diathesis has been suggested (346), on the basis of homology between the amino acid sequence of plasminogen and a 20-residue region of the dengue E glycoprotein. Cross-reactive antibodies between the dengue E peptide and the corresponding site of plasminogen were demonstrated in sera of dengue patients.

Why only a small subset (approximately 3%) of persons with secondary infections develop DHF remains uncertain. Both virus-specified and host-related factors may operate. Virus strains may vary with respect to the presence of enhancing epitopes or other intrinsic virulence factors. In the Americas, dengue type 2 has been responsible for repeated outbreaks of classical dengue fever as well as by the explosive appearance of DHF in Cuba, Venezuela, and Brazil. This apparent paradox could be explained solely on the basis of the epidemiological events leading to the establishment of hyperendemic transmission of multiple serotypes and an increased incidence of sequential infections. However, circumstantial evidence from molecular analysis suggests that the dengue 2 strain responsible for the occurrence of severe disease may represent a variant with increased virulence for humans.

A comparison of the gene sequences of dengue type 2 viruses from the Caribbean region indicated that two distinct variants were cocirculating over a period of many years (463). One genetic variant represented the Puerto Rican strain, that had been introduced into the region in 1969 and has persisted in an endemic-epidemic pattern, associated with classical dengue fever. A second variant represented a strain first isolated in Jamaica in 1981. The Jamaican genotype was responsible for the DHF epidemics in Venezuela (1989) and Brazil (1990). Sequence homology between the Jamaican variant and virus strains from Southeast Asia suggested the original source of introduction. In contrast, the Puerto Rican genotype resembled contemporary virus strains from Polynesia, where for the most part disease was mild. These observations infer that the Jamaican genotype of dengue 2 is more likely to cause DHF than the Puerto Rican genotype. The virus responsible for the large DHF epidemic in Cuba in 1981 has recently been sequenced (185). The responsible agent was distinct from the Jamaican genotype, and more closely resembled older strains from Southeast Asia.

Several workers have noted an apparent increase in the severity of disease expression during the chronologic progression of dengue epidemics (479,480). For example, during the 1981 Cuban epidemic, the incidence of severe grades of DHF and the case-fatality rate increased with time and was highest in the later stages of the outbreak (282). During a recent epidemic of dengue fever in Queensland, the incidence of hemorrhagic phenomena rose as the epidemic progressed (535). These observations suggested that dengue viral virulence may have been enhanced by rapid passage through the human population. No comparisons of early and late virus strains by sequencing or virus growth in human cells or mosquitoes have yet been reported to support this hypothesis.

A preliminary analysis demonstrated variation in the ability of dengue virus strains to replicate in human peripheral blood mononuclear cells (PBLs) and higher replication of isolates from DHF patients than from dengue fever patients (272). A second study of dengue 2 isolates also demonstrated higher replication in PBLs of strains from DHF patients than those from dengue fever patients (392). These studies were performed in PBLs and in the presence of enhancing antibodies from donors unrelated to the subject from whom the virus isolate was obtained. In future research, it would be important to determine the replication of the virus strain from an individual patient in autologous mononuclear cells and serum antibodies and the results correlated with disease outcome.

Host genetic factors may influence severity of dengue infection. An association between HLA haplotype and disease expression was found in Cuban patients with DHF. HLA-B "blank," HLA-A1, and HLA-Cw1 were more frequent and HLA-A29 was less frequent than in normal control subjects (421). In Thailand, HLA-B "blank" and HLA-A2 were associated with DHF (85). In the Cuban epidemic, whites had a higher incidence of DHF than persons of the black race, a finding that could not be explained on the basis of a racial difference in the background of immunity (46). Among acquired factors that influence disease expression, nutrition and underlying diseases may play a role. The prevalence of malnutrition is lower in children with DHF patients than in the general population (547), possibly reflecting the requirement for robust immunologic responsiveness in the genesis of DHF. Chronic diseases such as sickle cell disease, diabetes mellitus, and bronchial asthma appear to increase the risk of developing severe disease (46).

Clinical Features

The clinical manifestations of DHF and DSS are described by Cohen and Halstead (91) and by Nimmannitya et al. (406). Dengue hemorrhagic fever is distinguished from dengue fever by the presence of fever, thrombocytopenia (platelet count $<100\times10^9$/L), and hemoconcentration (hematocrit increased by >20%). Dengue shock syndrome is a more severe form of the disease, characterized by hypotension (pulse pressure ≤20 mmHg, cold clammy extremities), or profound shock (Table 6). The disease initially presents in a manner similar to that of classic dengue fever (see section entitled "Dengue Fever," above) but progresses after 2 to 5 days to a rapidly progressive, severe form with prostration, restlessness, irritability, shock with cold clammy extremities, diaphoresis, circumoral and peripheral cyanosis, rapid respiration, rapid pulse, and hypotension. Spontaneous hemorrhages occur, including petechiae, ecchymoses, oozing from venipuncture sites, epistaxis, etc. Clinically relevant hemorrhage occurs in 10% to 15% of cases. Gastrointestinal hemorrhages and intracerebral bleeding may be life-threatening. Physical findings include skin hemorrhages, pleural effusions, changes in vital signs, and hepatomegaly. Jaundice is a rare manifestation. Neurologic abnormalities have been reported in some cases, the most frequent manifestation being encephalopathy (stupor, coma, convulsions, paresis), generally with normal cerebrospinal fluid. Cases meeting the

TABLE 6. *Clinical grades of dengue hemorrhagic fever*[a]

Grade	Hemo-concentration[b]	Spontaneous hemorrhage	Hypo-tension[c]	Shock
I	+	0	0	0
II	+	+	0	0
III	+	+/–	+	0
IV	+	+/–	+	+

[a]Defined by the World Health Organization.
[b]Hematocrit increased by ≥20%.
[c]Hypotension and pulse pressure >20 mm Hg, physical signs of impaired circulation.
From World Health Organization (601).

criteria for Reye's syndrome have been described. Laboratory abnormalities in DHF include elevated hematocrit and hypoproteinemia (reflecting the capillary leakage), thrombocytopenia, elevated serum transaminases, depression of complement (especially C3) and fibrinogen levels, and the presence of fibrin split products in plasma. The progression of shock is rapid; without physiologic treatment, up to 50% of patients with profound shock die. However, early recognition and appropriate treatment have resulted in overall case-fatality rates of under 1%.

Diagnosis

Geographic location and epidemiologic setting are important clues to the diagnosis. Dengue hemorrhagic fever has generally not occurred in areas affected by other viral hemorrhagic fevers. An exception is the recent appearance of DHF in South America, where individual cases of the disease resemble and have been confused clinically with yellow fever. Chikungunya occasionally produces an illness with minor hemorrhagic manifestations similar to those of classic dengue fever, but cases with severe hemorrhage, hemoconcentration, thrombocytopenia, and shock are very rare or do not occur. Nonviral causes must be considered in the individual case, including bacterial sepsis, scrub and epidemic typhus, leptospirosis, severe malaria, and typhoid fever.

Virus isolation from tissues of fatal cases is less often successful than in classic dengue (407). Techniques for virus isolation from blood of acutely ill patients are discussed in Dengue Fever, above. Serodiagnosis is possible, but identification of the infecting subtype is difficult because most cases of DHF occur in persons with prior dengue exposure. Secondary-type antibody responses characterized by high HI, CF, and ELISA antibody titers, which are broadly cross-reactive, are typical in cases of DHF. Various attempts to examine the patterns of antibody responses to individual dengue proteins have not revealed consistent differences between dengue and DHF patients. In general, antibodies to the nonstructural proteins, including NS1, NS3, and NS5 are more prominent in sera of patients with secondary infections (79,294).

Treatment

The World Health Organization has formulated specific guidelines for the management of cases (601). Principles of treatment are dictated by the need to closely monitor the patient's vital signs and hematocrit and to replace plasma volume by judicious fluid replacement. Oxygen should be administered; moreover, if disseminated intravascular clotting is documented, consideration may be given to heparin therapy. Blood transfusion is indicated only in the case of severe hemorrhage. Salicylates and hepatotoxic drugs should be avoided. Corticosteroids are widely used; evidence for their usefulness is conflicting, but some studies indicate that they are of no value (539).

Specific, antiviral therapy has not been extensively evaluated. An uncontrolled trial of interferon was conducted during the 1981 epidemic in Cuba, with some indication that deaths may have been averted. Although ribavirin shows *in vitro* activity against dengue virus, concentrations required for *in vivo* efficacy are not achievable without toxicity. A study of ribavirin in dengue-infected monkeys failed to show an antiviral effect (339).

Epidemiology

Nearly 3 million cases of DHF have been officially reported world-wide through 1990, most cases occurring in Asia. In Thailand alone; 874,207 cases of DHF were reported between 1958 and 1990, with a case-fatality rate of 1.57%. In this country the disease ranks fifth as a cause of

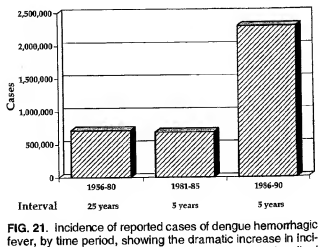


FIG. 21. Incidence of reported cases of dengue hemorrhagic fever, by time period, showing the dramatic increase in incidence in the last decade. The factors responsible are described in the text and include changes in human and vector ecology leading to the increased intensity and geographic expansion of virus transmission, and cocirculation of multiple dengue serotypes.

morbidity, third as a cause of death, and fifth as a cause of years of productive life lost (165). Factors responsible for the emergence of DHF as an epidemic disease in Asia in the 1950s and 1960s and for the dramatic rise in incidence in the last decade (Fig. 21) include:

1. Demographic changes (human population growth and urbanization) favoring contact with the domestic mosquito vector, *Aedes aegypti*.
2. Ecologic changes linked to urbanization (poor sanitation, inadequate piped water, necessitating domestic water storage) favoring *Aedes aegypti* breeding;
3. The rapid rise in air travel, providing the means for movement of viremic human beings and dissemination of multiple dengue serotypes.
4. Establishment of hyperendemic dengue infection and increasing frequency of sequential infections of children (176,388).

The factors responsible for the emergence of DHF in Asia have come to bear on the American region in the last 20 years. The distribution of *Aedes aegypti* changed dramatically, due to the collapse of mosquito control efforts (Fig. 16). The pattern of dengue infections changed from outbreaks at intervals of multiple years caused by a single serotype to annual outbreaks and cocirculation of three of the four dengue serotypes. Sporadic cases of DHF were recognized in the 1970s, and major epidemics occurred in Cuba, Venezuela, and Brazil in the 1980s. Eleven countries in the region have now reported the occurrence of DHF (Fig. 22).

Dengue hemorrhagic fever in Asia is a disease of childhood. Two peaks have been noted in age-specific incidence rates: children under 1 year old and children 3 to 5 years of age. The disease in infants is associated with primary infection in the presence of maternal antibody, whereas the vast majority of cases in older children is the result of secondary infections. Studies in Thailand have estimated the frequency of DHF and DSS in children 1 to 14 years of age to be 31 and 11 cases per 1,000 secondary dengue infections, respectively, whereas the incidence of DHF and DSS in primary infections is 1.9 and 0.07 per 1,000, respectively (62). An age-dependent excess in cases of severe DHF with shock syndrome in girls, compared with boys, has been noted and appears to be related to host factors, since serosurveys have shown no difference in sex-specific antibody prevalence.

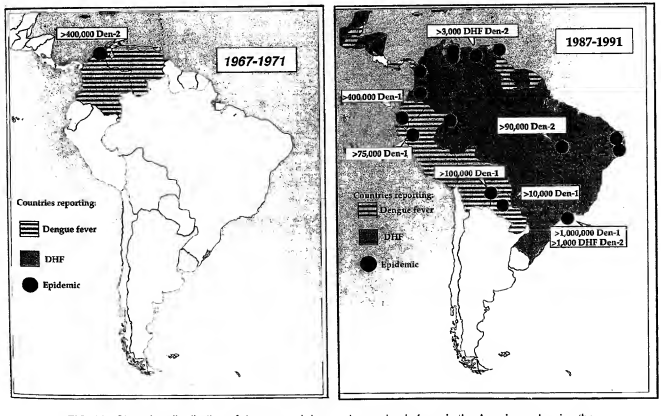


FIG. 22. Changing distribution of dengue and dengue hemorrhagic fever in the Americas, showing the spread of both diseases due to reinvasion of the South American continent by the *Aedes aegypti* vector and the introduction and transmission of multiple dengue serotypes. Outbreaks of DHF occurred in Brazil and Venezuela after introduction of dengue type 2 into a human populaton sensitized by prior outbreaks of dengue type 1.

The transmission cycle of dengue viruses has been described above (see Dengue Fever).

Prevention and Control

See Dengue Fever, above.

Other Flaviviruses Associated with Hemorrhagic Fever

Kyasanur Forest Disease Virus

Kyasanur Forest virus was isolated from a sick monkey (*Presbytis entellus*) in the Kyasanur Forest, Shimonga District, Karnataka (then Mysore) State, India, in 1957 (20, 599). The virus belongs to the TBE virus antigenic complex. No antigenic differences between strains have been found. The virus is lethal to infant and weanling mice by both the intracerebral and intraperitoneal routes, produces CPE or plaques in chick embryo, hamster, and monkey kidney cell cultures, and replicates without CPE in a continuous cell line of *Haemaphysalis spinigera* tick cells. Pathologic findings in human patients include parenchymal degeneration of the liver and kidneys, hemorrhagic pneumonitis, and an increase in reticuloendothelial tissue in liver and spleen, with marked erythrophagocytosis (337). Similar changes have been seen in experimentally infected monkeys. Bonnet macaques are susceptible to severe or lethal infections and demonstrate necrosis of lymphoid tissues (269). Some primate species also develop encephalitic lesions (chromatolysis of neurons and focal demyelination) (578). Lactating monkeys shed small amounts of virus in their milk. The clinical illness in humans is characterized by fever, headache, myalgia, cough, bradycardia, dehydration, hypotension, gastrointestinal symptoms, and hemorrhages (425). In some patients a biphasic course is seen; the first phase, as described above, lasts 6 to 11 days, followed by an afebrile period of 9 to 21 days and then the reappearance of fever and signs of meningoencephalitis. In such patients, the disease closely resembles Central European TBE (580). Leukopenia is a frequent finding during the acute phase of illness, and serum transaminase levels are raised. The cause of hemorrhage is unknown, but disseminated intravascular coagulation has been suspected. Diagnosis is by virus isolation from blood or serology. Standard serological tests and enzyme-linked immunoassays are applicable. Viremia is detected between the second and twelfth days of illness and is maximum between the third and sixth days, with a mean titer of 3.0 dex/mL. The disease is limited to Mysore State, India, but is gradually spreading. Epizootics occur in wild monkeys. Human infections occur principally during the dry season and in persons with close contact with forested areas. Thousands of cases have been reported since the recognition of the disease in 1957; the annual incidence of virologically diagnosed cases varies between 400 and 500 cases. The case-fatality rate is 3% to 5%; no sequelae are reported. The basic transmission cycle involves ixodid ticks and wild vertebrates, principally rodents and insectivores (20). Bats and ground-dwelling birds may play a role in transmission. Large animals (goats, cows, sheep) become infected, but viremias are low and their importance is principally as hosts sustaining tick populations. At least ten species of ixodid ticks have been implicated in transmission; *Haemaphysalis spinigera* is a major vector. Transtadial and transovarial transmissions occur in ticks. The density of tick vectors in a given year correlates with the incidence of human disease. A formalin inactivated vaccine produced in chick embryo fibroblasts has been licensed and is currently in use in the endemic area.

Omsk Hemorrhagic Fever Virus

Omsk hemorrhagic fever (OHF) virus was first isolated in 1947 from the blood of a patient with hemorrhagic fever during an epidemic in Omsk and Novosibirsk Oblasts, USSR. As demonstrated by serological relationships and nucleotide sequence homology (172), OHF virus is a member of the TBE virus complex. Strain heterogeneity has been noted in virulence and antigenic characteristics. The virus is pathogenic for infant and weanling mice by all routes of inoculation. Guinea pigs inoculated by the subcutaneous route develop fever and scattered deaths; rabbits develop antibodies only. Unlike Kyasanur Forest disease, OHF virus does not induce disease or significant pathology in experimentally inoculated bonnet macaques (269). The virus causes hemorrhagic disease and death in experimentally inoculated muskrats and narrow-skulled voles (*Microtus gregalis*). The virus has been propagated in pig kidney, Hela, and Detroit G cells. The disease in humans closely resembles Kyasanur Forest disease except that sequelae (hearing loss, hair loss, neuropsychiatric complaints) are relatively frequent. The case-fatality rate is 0.5% to 3%. Between 1945 and 1958 a total of nearly 1,500 cases were recorded in the Omsk region (270). Small numbers of cases acquired by tick bite continue to occur among rural residents engaged in field work during the spring and summer. Muskrat hunters are at risk of acquiring the disease by direct contact with blood and tissues, and such cases may occur during the winter months.

Muskrats were imported into the lake district of western Siberia from North America during the early 1900s, are susceptible to lethal infection, and experience epizootic die-offs. The basic transmission cycle remains uncertain. The ixodid tick vector *Dermacentor reticulatus* has been incriminated by field evidence and experimental infection (270). Other species, especially *Ixodes apronophorus*, are suspected to play a role in virus maintenance. Rodents, particularly water voles (*Arvicola terrestris*), are the principal viremic hosts. Direct rodent-to-rodent transmission

may occur. Muskrats are epizootic hosts, and human infections occur by direct contact with urine, feces, or blood. Virus isolations have also been made from several mosquito species, from a gamasid mite, and from sentinel mice. No specific Omsk hemorrhagic fever vaccine has been developed, but TBE vaccines apparently provide cross-protective immunity and have been used in high-risk population groups.

REFERENCES

1. Aaskov JG, Phillips DA, Wiemers MA. Possible clinical infection with Edge Hill virus. *Trans Roy Soc Trop Med Hyg* 1993;87:452–453.
2. Abdel Wahab KSE. Arboviruses and central nervous system disorders in Egypt. *Acta Virol (Praha)* 1970;14:501–506.
3. Aitken THG, Tesh RB, Beaty BJ, et al. Transovarial transmission of yellow fever virus by mosquitoes *(Aedes aegypti). Am J Trop Med Hyg* 1979;28:119–121.
4. Albagali C, Chaimoff R. A case of West Nile myocarditis. *Harefuah* 1959;57:274–275.
5. Albrecht P. Pathogenesis of neurotropic arbovirus infection. *Curr Top Microbiol Immunol* 1968;43:45–91.
6. Allan BC, Doherty RC, Whitehead RH. Laboratory infections with arboviruses including reports of two infections with Kunjin virus. *Med J Aust* 1966;2:844–850.
7. Allison S, Schalich J, Stiasny K, et al. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by acidic pH. (1994;in preparation).
8. Andersen AA, Hanson RP. Influence of sex and age on natural resistance to St. Louis encephalitis virus in mice. *Infect Immun* 1974;9: 1123–1125.
9. Anderson SG, Donnelley M, Stevenson WJ, et al. Murray Valley encephalitis: surveys of human and animal sera. *Med J Aust* 1952;1: 110–120.
10. Anonymous. Fatal viral encephalitis following 17D yellow fever vaccine inoculation: report of case in a 3-year old child; a joint statement. *JAMA* 1966;198:671–672.
11. Anonymous. Dengue and dengue hemorrhagic fever. *WHO Wkly Epidem Rec* 1993;68:357–360.
12. Anonymous. Yellow fever virus surveillance in Western Africa. *WHO Wkly Epidem Rec* 1994;69:93–95.
13. Anonymous. Inactivated Japanese encephalitis virus vaccine. *Morb Mort Wkly Rep* 1993;42:1–15.
14. Arsob H. Powassan encephalitis. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol IV. Boca Raton, FL: CRC Press, 1988;29–50.
15. Artsob H, Spence L, Surgeoner G, et al. Isolation of *Francisella tularensis* and Powassan virus from ticks (Acari:Ixodidae) in Ontario, Canada. *J Med Entomol* 1984;21:165–168.
16. Badman RT, Campbell J, Aldred J. Arbovirus infections in horses—Victoria, 1984. *Commun Dis Intell* 1984;17:5.
17. Bailey CL, Eldridge BF, Hayes DE, et al. Isolation of St. Louis encephalitis virus from overwintering *Culex pipiens* mosquitoes.*Science* 1978;199:1346–1349.
18. Ballinger-Crabtree ME, Miller BR. *J Gen Virol* 1990;71:2115–2121.
19. Bancroft WH, Top FH Jr, Eckels KH, et al. Dengue-2 vaccine: virological, immunological, and clinical responses of six yellow fever immune recipients. *Infect Immun* 1981;31:698–703.
20. Banerjee K. Kyasanur Forest disease. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol III. Boca Raton, FL: CRC Press, 1988;93–116.
21. Bannova GG, Sarmanova ES, Karavanov AS, et al. Study of biological properties of tick-borne encephalitis virus strains isolated in different parts of the distribution area. *Vopr Virusol* 1982;1:41–45.
22. Barrett ADT, Mathews JH, Miller BR, et al. Identification of monoclonal antibodies that distinguish between 17D-204 and other strains of yellow fever virus. *J Gen Virol* 1990;71:13–18.
23. Barrett ADT, Gould EA. Comparison of neurovirulence of different strains of yellow fever virus in mice. *J Gen Virol* 1986;67:631–637.
24. Barrett AD, Monath TP, Cropp CB et al. Comparison of wild-type yellow fever virus by passage in HeLa cells. *J Gen Virol* 1990;71: 2301–2306.
25. Baqar S, Hayes CG, Murphy JR, et al. Vertical transmission of West Nile virus by *Culex* and *Aedes* mosquitoes. *Am J Trop Med Hyg* 1993; 48:757–762.
26. Bearcroft WGC. Zika virus infection experimentally induced in a human volunteer. *Trans R Soc Trop Med Hyg* 1956;50:442–448.
27. Ben-Nathan D, Feuerstein G. The influence of cold or isolation stress on resistance of mice to West Nile encephalitis. *Experientia* 1990;46: 285–290.
28. Benenson MW, Top FJ Jr, Gresso W. The virulence to man of Japanese encephalitis virus in Thailand. *Am J Trop Med Hyg* 1975;24: 974–980.
29. Bennett N McK. Murray Valley encephalitis, 1974: clinical features. *Med J Aust* 1976;2:446–450.
30. Besselaar TG, Blackburn NK. Antigenic analysis of West Nile virus strains using monoclonal antibodies. *Arch Virol* 1988;99:75–88.
31. Bhamarapravati N, Tuochinda P, Boonyapaknavik V. Pathology of Thailand hemorrhagic fever. V. A study of 100 autopsy cases. *Ann Trop Med Parasitol* 1967;61:500–510.
32. Bhamarapravati N, Yoksan S. Study of bivalent dengue vaccine in volunteers. *Lancet* 1989;1:1077.
33. Bhamarapravati N, Yoksan S, Chayaniyayothin T, et al. Immunization with a live, attenuated dengue-2-virus candidate vaccine (16681 PDK53): clinical, immunological, and biological responses in adult volunteers. *Bull WHO* 1987;65:189–195.
34. Bhatt PN, Jacoby RO. Genetic resistant to lethal flavivirus encephalitis. II. Effect of immunosuppression. *J Infect Dis* 1976;134:166–173.
35. Blackburn NK, Reyers F, Berry WJ, et al. Susceptibility of dogs to West Nile virus: a survey and pathogenicity trial. *J Comp Pathol* 1989; 100:59–66.
36. Blaskovic D, Pucekova G, Kubinyi L. An epidemiological study of tick-borne encephalitis in the Tribec region: 1956–1963. *Bull WHO* 1967;36:89–94.
37. Blok J, Henchal EA, Gorman BM. Comparison of dengue viruses and some other flaviviruses by cDNA-RNA hybridization analysis and detection of a close relationship between dengue virus serotype 2 and Edge Hill virus. *J Gen Virol* 1984;65:2173–2181.
38. Blok J, McWilliam M, Butler HC, et al. Comparison of a dengue-2 virus and its candidate vaccine derivative: Sequence relationships with the flaviviruses and other viruses. *Virology* 1992;187:573–590.
39. Bond JO, Hammon, W. McD. Epidemiological studies of possible cross-protection between dengue and St. Louis encephalitis arboviruses in Florida. *Am J Epidemiol* 1970;92:321–330.
40. Boonpucknavig S, Vuttiviroj O, Boonpucknavig V. Infection of young adult mice with dengue virus type 2. *Trans R Soc Med Hyg* 1981;75: 647–653.
41. Boughton CR, Hawkes RA, Naim HM. Illness caused by a Kokoberalike virus in south-eastern Australia. *Med J Austral* 1986;145:90–92.
42. Bowen GS, Monath TP, Kemp GE. Geographic variation among St. Louis encephalitis virus strains in the viremic responses of avian hosts. *Am J Trop Med Hyg* 1980;29:1411–1419.
43. Brandriss MW, Schlesinger JJ, Walsh EA, et al. Lethal 17D yellow fever encephalitis in mice. I. Passive protection by monoclonal antibodies to the envelope proteins of 17D yellow fever and dengue 2 viruses.*J Gen Virol* 1986;67:229–234.
44. Brandt WE, Cardiff RD, Russell PK. Dengue virions and antigens in brain and serum of infected mice. *J Virol* 1970;6:500–506.
45. Brandt WE. From the World Health Organization. Development of Dengue and Japanese encephalitis vaccines. *J Infect Dis* 1990;162: 577–583.
46. Bravo JR, Guzman MG, Kouri GP. Why dengue hemorrhagic fever in Cuba? 1. Individual risk factors for dengue hemorrhagic fever/dengue shock syndrome. *Trans Roy Soc Trop Med Hyg* 1987;81:816–820.
47. Bray M, Lai CJ. Dengue virus premembrane and membrane proteins elicit a protective immune response. *Virology* 1991;185:505–508.
48. Bray M, Lai C-J. Construction of intertypic chimeric dengue viruses by substitution of structural protein genes. *Proc Natl Acad Sci USA* 1991;88:10342–10346.
49. Bray M, Zhao B, Markoff L, et al. Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against fetal dengue virus encephalitis. *J Virol* 1989;63:2853–2856.

50. Breinl A. Clinical, pathological and experimental observations on the "mysterious disease"; a clinically aberrant form of acute poliomyelitis. *Med J Aust* 1918;1:209–240.
51. Brès P. Historical review of dengue-1: implications of its introduction in the Western Hemisphere in 1977. In: *Dengue in the Caribbean, 1977*. Scientific Publication No. 375, Washington, DC: Pan American Health Organization, 1979;4–10.
52. Brinker KR, Paulson G, Monath TP. St. Louis encephalitis in Ohio, September, 1975: clinical and EEG studies in 16 cases. *Arch Intern Med* 1979;139:561–566.
53. Brinton MA. Characterization of West Nile virus persistent infections in genetically resistant and susceptible mouse cells. I. Generation of defective nonplaquing virus particles. *Virology* 1982;116:84–94.
54. Brinton MA, Dispoto JH. Sequence and secondary structure analysis of the 5'-terminal region of flavivirus genome RNA. *Virology* 1988; 162:290–299.
55. Brotherston JG, Boyce JB. Development of a non-infective protective antigen against louping-ill (schovirus group B): laboratory experiments. *J Comp Pathol* 1970;80:377–388.
56. Buescher, EL, Scherer WF. Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. *Am J Trop Med Hyg* 1959;8:719–722.
57. Bukowski JF, Kurane I, Lai CJ, et al. Dengue virus-specific cross-reactive CD8+ human cytotoxic T lymphocytes. *J Virol* 1989;63:5086–5091.
58. Buletsa BA, Turak IA, Korol M et al. [Neurologic manifestations of West Nile fever in the Transcarpathian region] *Zh Nevropatol Psikhiatr* 1989;89:29–30.
59. Burke DS, Lorsomrudee W, Leake CJ, et al. Fatal outcome in Japanese encephalitis. *Am J Trop Med Hyg* 1985;34:1203–1210.
60. Burke DS, Nisalak A. Detection of Japanese encephalitis virus immunoglobulin M antibodies in serum by antibody capture radioimmunoassay. *J Clin Microbiol* 1982;15:353–361.
61. Burke DS, Nisalak A, Gentry MK. Detection of flavivirus antibodies in human serum by epitope-blocking immunoassay. *J Med Virol* 1987; 23:163–173.
62. Burke DS, Nisalak A, Johnson DE. A prospective study of dengue infection in Bangkok. *Am J Trop Med Hyg* 1988;38:172–180.
63. Burke DS, Nisalak A, Ussery MA, et al. Kinetics of IgM and IgG responses in Japanese encephalitis virus in human serum and cerebrospinal fluid. *J Infect Dis* 1985;151:1093–1099.
64. Burke DS, Tingpalapong M, Ward GS, et al. Intensc transmission of Japanese encephalitis virus to pigs in a region free of epidemic encephalitis. *JE & HFRS Bull* 1986;1:17–26.
65. Burke T. Dengue hemorrhagic fever: a pathological study. *Trans R Soc Trop Med Hyg* 1968;62:682–692.
66. Calisher CH, Karabatsos N, Dalrymple JM, et al. Antigenic relationships among flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 1989;70:37–43.
67. Calisher CH, Poland JD. Laboratory diagnosis. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980;571–601.
68. Cammack N, Gould EA. Topographical analysis of epitope relationships on the envelope glycoprotein of yellow fever 17D vaccine and the wild-type Asibi virus. *Virology* 1986;150:333–341.
69. Cardiff RD, Lund JK. Distribution of dengue-2 antigens by electron immunocytochemistry. *Infect Immun* 1976;3:1699–1709.
70. Cardosa MJ, Porterfield JS, Gordon S. Complement receptor mediates enhanced flavivirus replication in macrophages. *J Exp Med* 1983;158: 258–263.
71. Carey DE. Chikungunya and dengue: a case of mistaken identity? *J Hist Med* 1971;26:243–262.
72. Casals J. The arthropod-borne group of animal viruses. *Trans NY Acad Sci* 1957;19:219–235.
73. Casals J, Brown LV. Hemagglutination with arthropod-borne viruses. *J Exp Med* 1954;99:429–449.
74. Cecilia D, Gould EA. Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. *Virology* 1991;181:70–77.
75. Chamberlain RW. History. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980;3–61.
76. Chamberlain RW, Kissling RE, Stamm DD, et al. Virus of St. Louis encephalitis in three species of wild birds. *Am J Hyg* 1957;65:110–118.
77. Chang GJ, Trent DW, Gubler DJ, Cheu M, Chi M. Phylogenetic and nucleotide signature patterns of the E protein gene of dengue 1 viruses de fine the geographic distribution and expansion of two E-genotypes. (manuscript).
78. Chang GJ, Cropp CB, Kinney RM, Trent DW, Gubler DJ. Nucleotide sequence variation of envelope protein gene identifies two distinct genotypes of yellow fever viruses. *J Virol* (submitted).
79. Chardboonchart V, Bhamarapravati N, Peampramprecha S, et al. Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. *Am J Trop Med Hyg* 1991;44:481–193.
80. Chardboonchart V, Khemarunmanus M, Yoksan S, et al. Dengue virus serotypic identification using suckling mouse and Western blot technique. *SE Asian J Trop Pub Hlth* 1990;21:614–620.
81. Chaturvedi UC, Mathur A, Chandra A, et al. Transplacental infection with Japanese encephalitis virus. *J Infect Dis* 1980;141:712–715.
82. Chaturvedi UC, Mukerjee R, Dhawan R. Active immunization by a dengue virus-induced cytokine. *Clin Exp Immunol* 1994;96:202–207.
83. Chen WR, tesh RB, Rico-Hesse R. Genetic variation of Japanese encephalitis in nature. *J Gen Virol* 1990;71:2915–2922.
84. Chen WR, Rico-Hesse R, Tesh RB. A new genotype of Japanese encephalitis virus from Indonesia. *Am J Trop Med Hyg* 1992;47:61–69.
85. Chieswlip P, Scott RM, Bhamarapravati N. Histocompatability antigens and dengue hemorrhagic fever. *Am J Trop Med Hyg* 1981;30:1100–1105.
86. Chumakov MP, Rubin SG, Semashko IV, et al. New perspective vaccines from tick-borne encephalitis virus propagated in green monkey kidney cell cultures. *Arch Virol* 1990;Suppl 1:161–168.
87. Chunikhin SP, Kurenkov VB. Viremia in *Clethrionomye glareolus*: a new ecological marker of tick-borne encephalitis virus. *Acta Virol (Praha)* 1979;23:257–260.
88. Clarke DH. Antigenic analysis of certain group B arthropod-borne viruses by antibody absorption. *J Exp Med* 1960;111:21–32.
89. Clarke DH. Further studies on antigenic relationships among the viruses of the group B tick-borne complex. *Bull WHO* 1964;31:45–56.
90. Coetzer JAW, Barnard BJH. Hydrops amnii in sheep associated with hydranencephaly and anthrogryphosis with wesselsbron disease and Rift Valley fever viruses as aetiological agents. *Onderstepoort J Vet Res* 1977;44:119–126.
91. Cohen SN, Halstead SB. Shock associated with dengue infection. I. The clinical and physiological manifestations of dengue hemorrhagic fever in Thailand, 1964. *J Pediatr* 1966;68:448–456.
92. Coia G, Parker MD, Speight G, et al. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. *J Gen Virol* 1988;69:1–21.
93. Conway D, Rossier E, Spence L, et al. Powassan virus encephalitis with shoulder girdle involvement. *Can Dis Weekly Rep* 1976;85:2.
94. Cordellier R. L'epidemiologie de la fievre jaune en Afrique de l'Ouest. *Bull WHO* 1991;69:73–84.
95. Cordellier R, Bouchite B, Roche JC, et al. Circulation selvatique du virus dengue 2 en 1980 dans les savanes sub-soudaniennes de Cote d'Ivoire. *Cah ORSTOM Ser Ent Med Parasit* 1983;21:165–179.
96. Corwin A, Habib M, Watts D, et al. Community-based prevalence profile of arboviral, rickettsial, and hantaan-like viral antibody in the Nile River Delta of Egypt. *Am J Trop Med Hyg* 1993;48:776–783.
97. Cruse RP, Rothner AD, Frenberg G, et al. Central European tick-borne encephalitis: an Ohio case with a history of foreign travel. *Am J Dis Child* 1979;133:1070–1071.
98. Cypress RH, Lubiniecki AS, Hammon W McD. Immunosuppression and increased susceptibility to Japanese B encephalitis virus in *Trichinella spiralis* infected mice. *Proc Soc Exp Biol Med* 1973;130:359–363.
99. Dalgarno L, Trent DW, Strauss JH, et al. Partial nucleotide sequence of the Murray Valley virus genome. *J Mol Biol* 1986;187:309–323.
100. Darnell MB, Koprowski H. Genetically determined resistance to infection with group B arboviruses. II. Increased production of interfering particles in cell cultures from resistant mice. *J Infect Dis* 1974;129: 248–256.
101. Darwish MA, Ibrahim AH. Prevalence of anibodies to arboviruses in Egypt: results of a serologic survey among 1,113 university students. *Am J Trop Med Hyg* 1975;24:981–985.
102. Das SK, Nityand S, Sood K, et al. Japanese B encephalitis with neurocysticercosis. *J Assoc Phys India* 1991;39:643–644.
103. Davidson MM, Williams H, MacLoed JA. Louping ill in man: a forgotten disease. *J Infect* 1991;23:241–249.
104. Davies DR, Padlan E-A. Antibody-antigen complexes. *Ann Rev Biochem* 1990;59:439–473.
105. Day JF. The influence of host health on mosquito engorgement success. *PhD dissertation*, University of Massachusetts, Amherst, 1982.

106. De Madrid AT, Porterfield JS. The flaviviruses (group B arboviruses): a cross-neutralization study. *J Gen Virol* 1974;23:91–96.
107. Defenda C, Staropoli I, Frenkiel MP, et al. Analysis of C-terminally truncated dengue 2 and dengue 3 virus envelope glycoproteins: in insect cells and immunologic properties in mice. *J Gen Virol* 1995;(in press).
108. Deubel V, Digoutte JP, Monath TP, et al. Genetic heterogeneity of yellow fever virus strains from Africa and the Americas. *J Gen Virol* 1986;67:209–213.
109. Deubel V, Ekue EK, Diop MM, et al. Introduction a l'analyse de la virulence du virus de la fievre jaune: etudes immunochemiques et biologiques comparatives entre les souches parentales. *Ann Inst Pasteur Virol* 1986;137E:1–11.
110. Deubel V, Kinney RM, Trent DW. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica: comparative analysis of the full-length genome. *Virology* 165:234–244.
111. Deubel V, Schlesinger JJ, Digoutte JP, et al. Comparative immunochemical and biological analysis of African and South American yellow fever viruses. *Arch Virol* 1987;94:331–338.
112. Deubel V, Laille M, Hughnot JP, Chungue E, Guesdon JE, Drouet MT, Bassot S, Chevrier D. Identification of dengue sequences by genomic amplification: rapid diagnosis of dengue virus serotypes in peripheral blood. *J Virol Meth* 1990;30:41–54.
113. Deubel V, Nogueira M, Drouet MT, et al. Direct sequencing of genomic cDNA fragments amplified by the polymerase chain reaction for molecular epidemiology of dengue 2 viruses. *Arch Virol* 1993;129: 197–210.
114. DeVries RRF, Meera Khan P, Bernin LE, et al. Genetic control of survival in epidemics. *J Immunogenet* 1980;6:271–287.
115. Dhawan R, Khanna M, Chaturvedi UC, et al. Effect of dengue virus-induced cytotoxin on capillary permeability. *J Exp Pathol* 1990;71:83–88.
116. Dickerson RB, Newton JR, Hansen JE. Diagnosis and immediate prognosis of Japanese B encephalitis. *Am J Med* 1952;12:277–290.
117. Doherty RL. Arthropod-borne viruses in Australia, 1973–1976. *Aust J Exp Biol Med Sci* 1977;55:103–130.
118. Doherty RL, Carley JG, Filippich C, et al. Murray Valley encephalitis in Australia, 1974: antibody response in cases and community. *Aust NZ J Med* 1976;6:446–453.
119. Doherty RL, Carley JG, Mackerras MJ, et al. Studies of arthropod-borne virus infections in Queensland. III. Isolation and characterization of virus strains from wild caught mosquitoes in North Queensland. *Aust J Exp Biol Med Sci* 1963;41:17–39.
120. Doherty RL, Carley JG, Kay BH, Filippich C, Marks EN, Frazier CL. Isolation of virus strains from mosquitoes collected in Queensland, 1972–1976. *Aust J Exp Biol Med Sci* 1979;57:509–520.
121. Doherty RL, Marks EM, Carley JG, Mackerras MJ. Studies of arthropodborne virus infections in Queensland. III. Isolation and characterization of virus strains from wild caught mosquitoes in north Queensland. *Aust J Exp Biol Med Sci* 1963;41:17–40.
122. Drewry SJ, Sanford JP, Luby JP, Unger RH, Kapland NM. Pathophysiology of epidemic St. Louis encephalitis. II. Pituitary-adrenal function. *Ann Intern Med* 1967;791:691–702.
123. Dropulic B, Masters CL. Entry of neurotropic arboviruses into the central nervous system: an in vitro study using mouse brain endothelium. *J Infect Dis* 1990;161:685–691.
124. Dunster LM, Gibson CA, Stephenson JR. Attenuation of virulence of flaviviruses following passage in HeLa cells. *J Gen Virol* 1990;71: 601–607.
125. Eckels KH, Dubois DR, Summers PL, et al. Immunization of monkeys with baculovirus-dengue type 4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection. *Am J Trop Med Hyg* 1994;50:472–478.
126. Edelman R, Nimmannitya S, Colman RW, et al. Evaluation of the plasma kinin system in dengue hemorrhagic fever. *J Lab Clin Med* 1975; 86:410–421.
127. Edman JD and Speilman A. Blood-feeding by vectors: physiology, ecology, behavior and vertebrate defense. In: Monath TP, ed. *The arboviruses: epidemiology and ecology*, vol I. Boca Raton, FL: CRC Press, 1988;153–189.
128. Edward DG. Immunization against louping-ill. Immunization of man. *Br J Exp Pathol* 1948;29:372–378.
129. Ehrenkranz NJ, Ventura AK, Cuadrado RR, et al. Pandemic dengue in Caribbean countries and the southern United States—past, present, and potential problems. *N Engl J Med* 1971;285:1460–1469.
130. Elbert LB, Krasilnikov IV, Drozdov SG, et al. Concentrated purified vaccine against tick-borne encephalitis produced by ultrafiltration and chromotography. *Vopr Virusol* 1985;1:90–93.
131. Falconar AKI, Young PR. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structrual glycoprotein NS1. *J Gen Virol* 1991;72: 961–965.
132. Falconar AKI, Young PR. Immunoaffinity purification of native dimer forms of the flavivirus non-structural glycoprotein, NS1. *J Virol Meth* 1990;30:323–332.
133. Falker WA, Diwan AR, Halstead SB. Human antibody to dengue soluble complement-fixing (SCF) antigens. *J Immunol* 1973;111: 1804–1809.
134. Feighny R, Burrous J, Putnak R. Dengue type-2 virus envelope protein made using recombinant baculovirus protects mice against virus challenge. *Am J Trop Med Hyg* 1994;50:322–328.
135. Figueiredo LTM, Cavalcante SMB, Simoes MC. Serological survey of dengue among schoolchildren in Rio de Janeiro, Brazil 1986 and 1987. *Bull PAHO* 1991;111:525–533.
136. Finley K, Riggs N. Convalescence and sequelae. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980;535–550.
137. Flatau E, Kohr D, Daker O, Varsano N. West Nile fever encephalitis. *Isr J Med Sci* 1981;17:1057–1059.
138. Flynn LM, Coelen RJ, Mackenzie JS. Kunjin virus isolates of Australia are genetically homogeneous. *J Gen Virol* 1989;70:2819–2824.
139. Fonseca BAL, Pincus S, Shope RE, et al. Recombinant vaccinia viruses co-expressing dengue-1 glycoproteins prM and E induce neutralizing antibodies in mice. *Vaccine* 1994;in press.
140. Forattini OP, Gomes A de C, Galati EAB, et al. Estudos ecologicos sobre mosquitos Culicidae no sistema da Serra do Mar, Brasil. *Rev Saude Publica* 1978;12:297–325.
141. French EL. Murray Valley encephalitis: isolation and characterization of the aetiological agent. *Med J Aust* 1952;1:100–103.
142. French EL. A review of arthropod-borne virus infections affecting man and animals in Australia. *Aust J Exp Biol Med Sci* 1973;51:131–158.
143. French EL, Anderson SG, Price AVG, et al. Murray Valley encephalitis in New Guinea. I. Isolation of Murray Valley encephalitis virus from the brain of a fatal case of encephalitis occurring in a Papuan native. *Am J Trop Med Hyg* 1957;6:827–834.
144. Fujita H, Sumiyoshi H, Mori C, et al. Studies in the development of Japanese encephalitis vaccine: expression of the envelope glycoprotein V3 (E) gene in yeast. *Bull WHO* 1987;65:303–308.
145. Gahlinger PM, Reeves WC, Milby MM. Air conditioning and television as protective factors in arboviral encephalitis. *Am J Trop Med Hyg* 1986;35:601–610.
146. Gaidamovich SY, Sokhey IU. Studies on antigenic peculiarities of West Nile virus strains isolated in the U.S.S.R. by three serological tests. *Acta Virol (Praha)* 1973;17:343–350.
147. Gao GF, Hussain MH, Reid HW, et al. Classification of a new member of the TBE flavivirus subgroup by its immunological, pathogenetic and molecular characteristics: identification of subgroup-specific pentapeptides. *Virus Res* 1993,30:129–144.
148. Gao GF, Hussain MH, Reid HW, Gould EA. Identification of naturally occurring monoclonal antibody escape variants of louping ill virus. *J Gen Virol* 1994;75:609–614.
149. Gardner JJ, Reyes MG. Pathology. In: Monath TP, ed. *Louis encephalitis*. Washington, DC: APHA, 1980;551–570.
150. Gateff C. Acquisitions récentes en matière d'associations vaccinales. *Bull Soc Pathol Exot Filiales* 1972;65:784–794.
151. Gatus BJ, Rose MR. Japanese B encephalitis: epidemiology, clinical and pathological aspects. *J infect* 1983;6:213–218.
152. Georges AJ, Lesbordes JL, Georges-Courbot MC, et al. Fatal hepatitis from West Nile virus. *Ann Inst Pasteur Virol* 1988;138:237–244.
153. Germain M, Comet M, Mouchet J, et al. Recent advances in research regarding sylvatic yellow fever in west and central Africa. *Bull Inst Pasteur* 1982;80:315–330.
154. Goldblum N. West Nile fever in the Middle East. *Proc 6th Int Cong Trop Med Parasitol* 1959;5:112–125.
155. Goldfarb LG. Epidemiological models of tick-borne infections (Acari: Ixodidae and Argasidae). *J Med Entomol* 1986;23:125–131.
156. Gollins SW, Porterfield JS. pH-Dependent fusion between the flavivirus West Nile and lysosomal model membranes. *J Gen Virol* 1986;67: 157–166.

157. Gollins SW, Porterfield JS. Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. *J Gen Virol* 1984;65:1261–1272.
158. Gomes G, Causey OR, Bussuquara, a new arthropod-borne virus. *Proc Soc Exp Biol Med* 1939;101:273–279.
159. Goodman GT, Koprowski H. Study of the mechanism of innate resistance to virus infection. *J Cell Comp Physiol* 1962;59:333–341.
160. Gould EA, Buckley A, Groeger BK, et al. Immune enhancement of yellow fever virus neurovirulence for mice: studies of mechanisms involved. *J Gen Virol* 1987;68:3105–3112.
161. Gould EA, Buckley A. Antibody dependent enhancement of yellow fever and Japanese encephalitis virus neurovirulence. *J Gen Virol* 1989; 70:1605–1608.
162. Gould EA, Buckley A, Barrett ADT, et al. Neutralizing (54 K) and non-neutralizing (54 K and 48 K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confers immunity in mice. *J Gen Virol* 1986;67:591–595.
163. Goverdhan MK, Kulkarni AB, Gupta AK, Tupe CD, Rodrigues JJ. Two-way cross-protection between West Nile and Japanese encephalitis viruses in bonnet macaques. *Acta Virol* 1992;36:277–283.
164. Gradoth N, Weitzman S, Lehmann EE. Acute anterior myelitis complicating West Nile fever. *Arch Neurol* 1979;36:172–173.
165. Gratz NG. Lessons of Aedes aegypti control in Thailand. *Med Vet Entomol* 1993;7:1–10.
166. Green S, Kurane I, Edelman R, et al. Dengue virus-specific human CD4+ T-lymphocyte responses in a recipient of an experimental live-attenuated dengue virus type 1 vaccine: Bulk culture proliferation, clonal analysis, and precursor frequency determination. *J Virol* 1993;67: 5962–5967.
167. Gresikova M, Beran GW. Tick-borne encephalitis. In: Beran GW, ed.*CRC handbook series in zoonoses, section B: viral zoonoses*, vol I. Boca Raton, FL: CRC Press, 1981:201–208.
168. Gresikova M, Calisher CH. Tick-borne encephalitis. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol IV. Boca Raton, FL: CRC Press, 1988;177–203.
169. Gresikova M, Sekeyova M, Stupalova S, et al. Sheep milk-borne epidemic of tick-borne encephalitis in Slovakia. *Intervirology* 1975;5: 57–61.
170. Gresikova-Kohutova, M. Effect of pH on infectivity of the tick-borne encephalitis virus. *Acta Virol* 1959;3:159–167.
171. Grimstad FR, Barrett CL, Humphrey RL, et al. Serologic evidence for widespread infection with LaCrosse and St. Louis encephalitis viruses in the Indiana human population. *Am J Epidemiol* 1987;119:913–930.
172. Gritsun TS, Lashkevich VA, Gould EA. Nucleotide and deduced amino acid sequence of the envelope glycoprotein of Omsk haemorrhagic fever virus; comparison with other flaviviruses. *J Gen Virol* 1993;74: 287–291.
173. Grossman RA, Edelman R, Gould DJ. Study of Japanese encephalitis virus in Chiangmai Valley Thailand. *Am J Epidemiol* 1974;100:69–76.
174. Gubler DJ. Current research on dengue. *Curr Top Vector Res* 1987;3: 37–56.
175. Gubler DJ. Dengue. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol II. Boca Raton, FL: CRC Press, 1988;223–260.
176. Gubler DJ, Trent DW. Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. *Infect Agents Dis* 1994;2:383–393.
177. Gubler DJ. Dengue/dengue hemorrhagic fever in the Americas: prospects for the year 2000. In: Halstead SB, Gomez-Dantes H, eds. *Dengue, a worldwide problem, a common strategy*. Proceedings of International Conference on Dengue and Aedes aegypti Community Based Control, Merida, Mexico, July 11–16, 1992. Mexico Minister of Health, Mexico City, 1992;19–27.
178. Gubler DJ, Kuno G, Waterman SH. Neurologic disorders associated with dengue. In: Pang T, Pathmanathan L, eds. *Proceedings of the international conference on dengue*. Kuala Lampur. University of Malaysia Press, 1984;290–300.
179. Gubler DJ, Suharyono W, Tan R, et al. Viraemia in patients with naturally acquired dengue infection. *Bull WHO* 1981;59:623–630.
180. Gubler DJ, Suharyono S, Wulur H, et al. Virological surveillance for dengue haemorrhagic fever in Indonesia using the mosquito inoculation technique. *Bull WHO* 1979;57:931–936.
181. Guillon JC, Oudar J, Joubert L, et al. Lesions histologiques due système nerveux dans l'infection a virus West Nile chez le cheval. *Ann Inst Pasteur* 1968;114:539–550.
182. Guirakoo F, Radda AC, Heinz FX, et al. Evidence for antigenic stability of tick-borne encephalitis virus by the analysis of natural isolates. *J Gen Virol* 1987;68:859–864.
183. Guirakhoo F, Heinz FX, Kunz C. Epitope model of tick-borne encephalitis virus envelope glycoprotein E: Analysis of structural properties, role of carbohydrate side chains, and conformational changes occurring at acidic pH. *Virology* 1989;169:90–99.
184. Guirakhoo F, Heinz FX, Mandl CW, et al. Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. *J Gen Virol* 1991;72:1323–1329.
185. Guzman MG, Deubel V, Pelegrino JL, et al. Partial nucleotide and amino acid sequence of the envelope and E/NS1 gene junction of four dengue 2 strains isolated during the 1981 DHF/DSS Cuban epidemic. *Am J Trop Med Hyg* 1995;52:241–246.
186. Habu A, Murakami Y, Ogasa A, Fujisaki Y. Disorder of spermatogenesis and viral discharge into semen in boars infected with Japanese encephalitis virus. *Virus* 1977;27:21–26.
187. Hahn CS, Dalrymple JM, Strauss JH, et al. Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *Proc Natl Acad Sci USA* 1987;84:2019–2023.
188. Hahn YS, Galler R, Hunkapiller T, et al. Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* 1988;162:167–180.
189. Halstead SB. *In vivo* enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J Infect Dis* 1979;140: 527–533.
190. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. *Science* 1988;239:476–481.
191. Halstead SB. Antibody, macrophages, dengue virus infection, shock and hemorrhage: a pathogenetic cascade. *Rev Infect Dis* 1989;11:S830–S839.
192. Halstead SB, Nimmannitya S, Cohen SN. Observations related to pathogenesis of dengue related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J Biol Med* 1970;42:311–328.
193. Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J Exp Med* 1977;146:201–217.
194. Halstead SB, Rojanasuphot S, Sangkawibha N. Original antigenic sin in dengue. *Am J Trop Med Hyg* 1983;32:154–156.
195. Halstead SB, Venkateshan CN, Gentry MK, et al. Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies. *J Immunol* 1984;132:1529–1532.
196. Hambleton P, Stephenson JR, Baskerville A, et al. Pathogenesis and immune response of vaccinated and unvaccinated rhesus monkeys to TBE virus. *Infect Immun* 1983;40:995–1003.
197. Hammon W McD. Observations on dengue fever, benign protector and killer: a Dr. Jekyll and Mr. Hyde. *Am J Trop Med Hyg* 1969;18: 159–165.
198. Hammon W McD, Sather GG. Immunity of hamsters to West Nile and Murray Valley encephalitis viruses following immunization with St. Louis and Japanese B. *Proc Soc Exp Biol Med* 1956;91:521–524.
199. Hanna C, Jarman RV, Keatts JG, Duffy CE. Virus-induced cataracts. *Arch Ophthalmol* 1968;79:59–63.
200. Harabacz I, Bock H, Jüngst C, et al. A randomized phase II study of a new tick-borne encephalitis vaccine using three different doses and two immunization regimes. *Vaccine* 1992;10:145–150.
201. Hardy JL. Susceptibility and resistance of vector mosquitoes. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol I. Boca Raton, FL: CRC Press, 1988;87–126.
202. Harrison AK, Murphy FA, Gardner JJ, et al. Myocardial and pancreatic necrosis induced by Rocio virus, a new flavivirus. *Exp Mol Pathol* 1980;32:102–113.
203. Hase T. Virus-neuron interactions in the mouse brain infected with Japanese encephalitis virus. *Virchows Arch B Cell Path Mol Pathol* 1993;64:161–170.
204. Hase T, Summers PL, Dubois DR. Ultrastructural changes of mouse brain neurons infected with Japanese encephalitis virus. *Int J Exp Pathol* 1990;71:493–505.
205. Hasegawa H, Yoshida M, Shiosaka T, et al. Mutations in the envelope protein of Japanese encephalitis virus affect entry into cultured cells and virulence in mice. *Virology* 1992;191:158–165.
206. Hawkes RA, Boughton CR, Naim HM, Wild J, Chapman B. Arbovirus infections of humans in New South Wales. Sero-epidemiology of the

flavivirus group of togaviruses. *Med J Austral* 1985;143:555–561.
207. Hawkes RA, Roehrig JT, Hunt AR, et al. Antigenic structure of the Murray Valley encephalitis virus E-glycoprotein. *J Gen Virol* 1988; 69:1105–1109.
208. Hawkes RA, Pamplin J, Boughton CR et al. Arbovirus infections of humans in high-risk areas of south-eastern Australia. *Med J Aust* 1993; 159:159–162.
209. Hawkes RA, Roehrig JT, Boughton CR et al. Defined epitope blocking with Murray Valley encephalitis virus and monoclonal antibodies: laboratory and field studies. *J Med Virol* 1990;32:31–38.
210. Hayashi K, Arita T. Experimental double infection of Japanese encephalitis virus and herpes simplex in mouse brain. *Jpn J Exp Med* 1977;47:9–13.
211. Hayes CG. West Nile Fever. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol. I. Boca Raton, FL: CRC Press, 1988;87–126.
212. Hearn HJ Jr, Soper WT, Miller WS. Loss in virulence of yellow fever virus serially passed in HeLa cells. *Proc Soc Exp Biol Med* 1965;1119: 319–322.
213. Heinz FX. Epitope mapping of flavivirus glycoproteins. *Adv Virus Res* 1986;31:103–168.
214. Heinz FX, Berge R, Tuma W, et al. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. *Virology* 1983;126:525–537.
215. Heinz FX, Kunz C. Protease treatment and chemical cross-linking of a flavivirus: tick-borne encephalitis virus. *Arch Virol* 1979;60:207–216.
216. Heinz FX, Kunz C, Fauma H. Preparation of a highly purified vaccine against tick-borne encephalitis virus by continuous-flow zonal ultracentrifugation. *J Med Virol* 1980;6:213–221.
217. Heinz FX, Kunz C. Molecular epidemiology of tick-borne encephalitis virus: peptide mapping of large non-structural proteins of European isolates and comparison with other flaviviruses. *J Gen Virol* 1982;62: 271–285.
218. Heinz FX, Mandl C, BergeR R, et al. Antibody-induced conformational changes result in enhanced avidity of antibodies to different antigenic sites on the tick-borne encephalitis virus glycoprotein. *Virology* 1984; 133:25–34.
219. Heinz FX, Mandl CW, Guirakhoo F, et al. The envelope protein E of tick-borne encephalitis virus and other flaviviruses: structure, functions and evolutionary relationships. *Arch Virol* 1990;Suppl 1:125–135.
220. Heinz FX, Mandl CW, Holzmann H, et al. The flavivirus envelope protein E: Isolation of a soluble form from Tick-borne encephalitis virus and its crystallization. *J Virol* 1991;65:5579–5583.
221. Heinz, FX, Roehrig JT. Flaviviruses. In: *Immunochemistry of viruses*, vol II. Amsterdam-New York-Oxford: Elsevier, 1990;289–305.
222. Heinz FX, Stiasny K, Puschner-Auer G, et al. Structural changes and functional control of the Tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. *Virology* 1994;198: 109–117.
223. Heinz FX, Tuma W, Kunz C. Antigenic and immunogenic properties of defined physical forms of tick-borne encephalitis virus structural proteins. *Infect Immun* 1981;37:250–257.
224. Henchal EA, Gentry MK, McCown JM, Brandt WE. Dengue virus specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am J Trop Med Hyg* 1982; 31:830–836.
225. Henchal EA, Henchal LS, Thaisomboonsuk BK. Topological mapping of unique epitopes on the dengue 2 virus NS1 protein using monoclonal antibodies. *J Gen Virol* 1987;68:845–851.
226. Henchal EA, McCown JM, Burke DS, et al. Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. *Am J Trop Med Hyg* 1985;34:162–169.
227. Henchal EA, Henchal LS, Schlesinger JJ. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with Dengue,2 virus. *J Gen Virol* 1988;69:2101–2107.
228. Henderson BE, Cheshire PP, Kirya GB, et al. Immunologic studies with yellow fever and selected African group B arboviruses in rhesus and vervet monkeys. *Am J Trop Med Hyg* 1970;19:110–118.
229. Henderson BE, Pigford CA, Work T, et al. Serologic survey for St. Louis encephalitis and other group B arbovirus antibodies in residents of Houston, Texas. *Am J Epidemiol* 1970;91:87–98.
230. Hill AB, Lobigs M, Blanden RV, et al. The cellular immune response to flaviviruses. In: Thomas DB, ed. *Viruses and the cellular immune response*. New York: Marcel Dekker, 1993;363–388.
231. Hill AB, M, Ilbacher A, Parrish C, et al. Broad cross-reactivity with marked fine specificity in the cytotxic T cell response to flaviviruses. *J Gen Virol* 1992;73:1115–1123.
232. Hober D, Poli L, Roblin B, et al. Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in dengue-infected patients. *Am J Trop Med Hyg* 1993;48:324–331.
233. Hofmann H, Kunz C, Heinz FX. Laboratory diagnosis of tick-borne encephalitis. *Arch Virol* 1990;Supp1 1:153–159.
234. Hoke CH Jr, Nisalak A, Sangawhipa N, et al. Protection against Japanese encephalitis by inactivated vaccines. *N Engl J Med* 1988;319: 608–613.
235. Hoke CH Jr, Vaughn DW, Nisalak A et al. Effect of high-dose dexamethasone on the outcome of acute encephalitis due to Japanese encephalitis virus. *J Infect Dis* 1992;165:631–637.
236. Holland J, Spindler K, Horodyski H, et al. Rapid evolution of RNA genomes. *Science* 1982;215:1577–1585
237. Holzmann H, Heinz FX, Mandl CW, et al. A single amino acid substitution in the envelope protein E of tick-borne encephalitis virus leads to attenuation in the mouse model. *J Virol* 1990;64:5156–5159.
238. Holzmann H, Utter G, Norrby E, et al. Assessment of the antigenic structure of Tick-borne encephalitis virus by the use of synthetic peptides. *J Gen Virol* 1993;74:2031–3025.
239. Holzmann H, Vorobyova MS, Ladyshenskaya JP, et al. Molecular epidemiology of tick-borne encephalitis virus: crossprotection between European and Far Eastern subtype. *Vaccine* 1992;10:345–349.
240. Huang CH. Studies of Japanese encephalitis in China. *Adv Virus Res* 1982;27:71–101.
241. Huang CH, Wong C. Relation of the peripheral multiplication of Japanese B encephalitis virus to the pathogenesis of the infection in mice. *Acta Virol (Praha)* 1963;7:322–330.
242. Huong VTQ, Ha DQ, Deubel V. Genetic study of Japanese encephalitis viruses from Vietnam. *Am J Trop Med Hyg* 1993;49:538–544.
243. Ilkal MA, Dhanda V, Hassan MM, et al. Entomological investigations during outbreaks of dengue fever in certain villages in Maharashtra State. *Indian J Med Res* 1991;93:174–178.
244. Ilkal MA, Dhanda V, Rao BU, et al. Absence of viaemia in cattle after experimantal infection with Japanese encephalitis virus. *Trans Roy Sco Trop Med Hyg* 1988;82:628–631.
245. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, et al. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 1989;40:418–427.
246. Innis BL, Thirawuth V, Hemachudha C. Identification of continuous epitopes of the envelope glycoprotein of dengue type 2 virus. *Am J Trop Med Hyg* 1989;40:676–687.
247. Iversson LB. Rocio encephalitis. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol IV. Boca Raton, FL: CRC Press, 1988; 77–92.
248. Iversson LB. Agestos da epidemia da encefalite por arbovírus na região do Valle do Ribeira, São Pulo, Brasil, no período de 1975 a 1978. *Rev Saúde Públ S Paulo* 1980;14:9–35.
249. Iversson LB, Coimbra TLM, Travassos da Rosa APA, et al. Use of immunoglobulin M antibody capture enzyme-linked immunosorbent assay in the surveillance of Rocio encephalitis. *Ciência Cult* 1992;44:164–166.
250. Jacoby RO, Bhatt PN. Genetic resistance to lethal flavivirus encephalitis. I. Infection of congenic mice with Banzi virus. *J Infect Dis* 1976; 134:158–165.
251. Jacoby RO, Bhatt PN, Schwartz A. Protection of mice from lethal flavivirus encephalitis by adoptive transfer of splenic cells from donors infected with Banzi virus. *J Infect Dis* 1980;141.617–624.
252. Jacobs SC, Stephenson JR, Wilkinson GWG. High-level expression of the Tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector: Protection elicited in a murine model. *J Virol* 1992;66: 2086–2095.
253. Jan LR, Yang CS, Henchal I.S, et al. Increased immunogenicity and protective efficacy in outbred and inbred mice by strategic carboxyl-terminal truncation of Japanese encephalitis virus envelope glycoprotein. *Am J Trop Med Hyg* 1993;48:412–423.
254. Jennings AD, Whitby JE, Minor PD, et al. Nucleotide and deduced amino acid sequence of the envelope protein of the wild-type French viscerotropic strain of yellow fever virus, and the French neurotropic vaccine strain derived from it. *Virology* 1993;192:692–695.
255. Jennings AD, Gibson CA, Miller BR, et al. Analysis of a yellow fever virus isolated from a fatal case of vaccine-associated human encepha-

litis. *J Infect Dis* 1994;169:512–518.
256. Jiang WR, Lowe A, Higgs S, et al. Single amino acid codon changes detected in Louping ill virus antibody resistant mutants induce reduced neurovirulence. *J Gen Virol* 1993;74:931–935.
257. Jimenez DR, Santana JL, Ramirez-Ronda CH. Neurological disorders associated to dengue infection. *Bol Assoc Med PR* 1988;80:208–211.
258. Johnson KM, Halstead SB., Cohen SN. Hemorrhagic fevers of Southeast Asia and South America: a comparative appraisal. *Prog Med Virol* 1967;9:105–158.
259. Johnson RT. *Viral infections of the nervous system*. New York: Raven Press, 1982.
260. Johnson RT, Burke DS, Elwell M, et al. Japanese encephalitis: immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. *Ann Neurol* 1985;18:567–573.
261. Kaplan JE, Eliason DA, Moore M. Epidemiologic investigations of dengue infection in Mexico, 1980. *Am J Epidemiol* 1983;117:335–343.
262. Karabatsos N. General characteristics and antigenic relationships. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980; 159–200.
263. Karabatsos N, ed. *International catalogue of arthropod-borne viruses*, 3rd ed., San Antonio, Texas: American Society for Tropical Medicine and Hygiene, 1985.
264. Kaufman BM, Summers PL, DuBois DR, et al. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. *Am J Trop Med Hyg* 1989;41:578–580.
265. Kawano H, Rostapshov V, Rosen L, et al. Genetic determinants of dengue type 4 virus neurovirulence for mice. *J Virol* 1993;67:6567–6575.
266. Kay BH, Carley JG, Fanning ID, Filippich C. Quantitative studies of the vector competence of *Aedes aegypti*, *Culex annulirostris* and other mosquitoes (Diptera: Culicidae) with Murray Valley encephalitis and other Queensland arboviruses. *J Med Entomol* 1979;16:59–66.
267. Kay BH, Fanning ID, Mottram P. Rearing temperature influences flavivirus vector competence of mosquitoes. *Med Vet Entomol* 1989;3: 415–422.
268. Kay BH, Saul AJ, McCullagh A. Mathematical model for rural amplification of Murray Valley encephalitis in southern Australia. *Am J Epidemiol* 1987;125:690–705.
269. Kenyon RH, Rippy MK, McKee KT Jr. Infection of *Macaca radiata* with viruses of the tick-borne encephalitis group. *Microb Pathog* 1992; 13:399-409.
270. Kharitonova NN, Leonov YuA. *Omsk hemorrhgic fever. Ecology of the agent and epizootology*. New Delhi: Amerind Publ Co, 1985.
271. Kliks SC, Halstead SB. An explanation for enhanced virus plaque formation in chick embryo cells. *Nature* 1980;285:504–505.
272. Kliks S. Antibody-enhanced infection of monocytes as the pathogenetic mechanism for severe dengue illness. *AIDS Res Hum Retrovir* 1990; 6:993–998.
273. Klockmann U, Bock HL, Franke V, et al. Preclinical investigations of the safety, immunogenicity and efficacy of a purified, inactivated tick-borne encephalitis vaccine. *J Biol Stand* 1989;17:331–342.
274. Ko Y-C, Chen M-J, Yeh S-M. The predisposing and protective factors against dengue virus transmission by mosquito vector. *Am J Epidemiol* 1992;136:214–220.
275. Kobayashi Y, Hasegawa H, Oyama T, et al. Antigenic analysis of Japanese encephalitis virus by using monoclonal antibodies. *Infect Immun* 1984;44:117–123.
276. Kobiler D, Lustig S, Gozes Y et al. Sodium dodecylsulphate induces a breach in the blood-brain barrier and enables a West Nile virus variant to penetrate into mouse brain. *Brain Res* 1989;496:314–316.
277. Konishi E, Pincus S, Fonseca BA, Shope RE, Paoletti E, Mason PW. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology* 1991;185:401–410.
278. Konishi E, Pincus S, Paoletti E, et al. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 1992;188: 714–720.
279. Konishi E, Pincus S, Paoletti E, et al. A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the prM, E, and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. *Virology* 1992;190:454–458.
280. Konishi E, Pincus S, Paoletti E, Shope RE, Burrage T, Mason PW. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 1992;188:714–720.
281. Kontny U, Kurane I, Ennis FA. Interferon gamma augments Fc receptor-mediated dengue virus infection of human monocytic cells. *J Virol* 1988;62:3928–3933.
282. Kouri GP, Guzman MG, Bravo JR. Why dengue hemorrhagic fever in Cuba? 2. An integral analysis. *Trans Roy Soc Trop Med Hyg* 1987;78: 821–823.
283. Krishnamurthi C, Alving B. Effect of dengue virus on procoagulant and fibrinolytic activities of monocytes. *Rev Infect Dis* 1989;11: S843–S846.
284. Kuberski TT, Rosen L. A simple method for the detection of dengue antigen in mosquitoes by immunofluorescence. *Am J Trop Med Hyg* 1977;26:533–537.
285. Kubes M, Fuchsberger N, Labuda M, et al. Salivary gland extracts of partially fed *Dermacentor reticulatus* ticks decrease natural killer cell activity *in vitro*. *Immunol* 1994;84:911–916 .
286. Kulkarni AB, M,Ibacher A, Parrish CR, et al. Analysis of murine major histocompatibility complex class II restricted T-cell responses to the flavivirus Kunjin by using vaccinia virus expression. *J Virol* 1992;66: 3583–3592.
287. Kumar R, Mathur A, Kumar A, et al. Virological investigations of acute encephalopathy in India. *Arch Dis Child* 1990;65:1227–1230.
288. Kumar R, Mathur A, Singh KB et al. Clinical sequelae of Japanese encephalitis in children. *Ind J Med Res* 1993;97:9–13.
289. Kundin WD, Liu C, Hysell P, et al. Studies in West Nile infection by means of fluorescent antibodies. I. Pathogenesis of West Nile virus infection experimentally inoculated in suckling mice. *Arch Ges Virusforsch* 1963;12:514–528.
290. Kuno S, Vorndam AV, Gubler DJ, et al. Study of anti-dengue NS1 antibody by Western Blot. *J Med Virol* 1990;32:102–108.
291. Kuno G, Gómez I, Gubler DJ. An ELISA procedure for the diagnosis of dengue infections. *J Virol Meth* 1991;33:101–113.
292. Kuno G, Gubler DJ, Oliver A. Use of 'original antigenic sin' theory to determine the serotypes of previous dengue infections. *Trans Roy Soc Trop Med Hyg* 1993;87:103–105.
293. Kuno G, Gubler DJ, Velez M, et al. Comparative sensitivity of three mosquito cell lines for isolation of dengue viruses. *Bull WHO* 1985; 63:279–286.
294. Kuno G, Vorndam AV, Gubler DJ, Gomez I. Study of anti-dengue NS1 antibody by Western blot. *J Med Virol* 1990;32:102–108.
295. Kunz C, Heinz FX, Hofmann H. Immunogenicity and reactigenicity of a highly purified vaccine against tick-borne encephalitis. *J Med Virol*, 1980;6:103–109.
296. Kunz C, Hofmann H, Kundi M, et al. Zur Wirksamkeit von FSME Immunglobulin. *Wiener Klin Wschr* 1981;93:665–667.
297. Kunz C, Hofmann H, Stary A. Field studies with a new tick-borne encephalitis (TBE) vaccine. *Zbl Bakt 1 Orig A* 1976;243:141–144.
298. Kunz C. Tick borne encephalitis in Europe. *Acta Leidensia* 1992;60-2:1–14
299. Kurane I, Brinton MA, Samson AL, et al. Dengue virus-specific human CD4+CD- cytotoxic T-cell clones: Multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. *J Virol* 1991;65: 1823–1928.
300. Kurane I, Dai L-C, Lingston PG, et al. Definition of an HLA-DPw2-restricted epitope on NS3, recognized by a dengue virus serotype-cross-reactive human CD4+CD8- cytotoxic T-cell clone. *J Virol* 1993;67: 6285–6288.
301. Kurane I, Innis BL, Nimmannitya S, et al. Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-α in sera of children with dengue. *J Clin Invest* 1991;88:1473–1480.
302. Kurane I, Innis BL, Nimmannitya S, et al. High levels of interferon alpha in the sera of children with dengue virus infection. *Am J Trop Med Hyg* 1993;48:222–229.
303. Kurane I, Innis BL, Nisalak A, et al. Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. *J Clin Invest* 1989;83:506–513.
304. Kurane I, Meager A, Ennis FA. Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon-α production and cytotoxic activity. *J Exp Med* 1989;170:763–775.
305. Kurane I, Ennis FA. Immunity and immunopathology in dengue virus infections. *Seminars in Immunol* 1992;4:121–127.
306. Kutubuddin M, Kolaskar AS, Galande S, et al. Recognition of helper T cell epitopes in envelope (E) glycoprotein of Japanese encephalitis,

West Nile and dengue virus. *Molec Immunol* 1991;28:149–154.
307. Labuda M, Jones LD, Williams T, Nuttall PA. Enhancement of tick-borne encephalitis virus transmission by tick salivary gland extracts. *Med Vet Entomol* 1993;7:193–196.
308. Labuda M, Jones LD, Williams T, Danielova V, Nuttall PA. Efficient transmission of tick-borne encephalitis virus between cofeeding ticks. *J Med Entomol* 1993;30:295–299.
309. Lai C-J, Zhao B, Hori H, et al. Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus. *Proc Natl Acad Sci USA* 1991;88:5139–5143.
310. Lanciotti RS, Calisher CH, Gubler DJ, et al. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992;30:545–551.
311. Lanciotti RS, Gubler DJ, Trent DW. Molecular evolution and phylogeny of dengue-4 viruses. *Virology* (submitted).
312. Lange DG, Sedmak J. Japanese encephalitis virus (JEV): potentiation of lethality in mice by microwave radiation. *Bioelectromagenetics* 1991; 12:335–348.
313. Leclerc C, Dériaud E, Megret F, et al. Identification of helper T cell epitopes of dengue virus E-protein. *Molec Immunol* 1993;30:613–625.
314. Lee E, Pemon C, Simpson R, et al. Sequence of the 3′: half of the Murray Valley encephalitis virus genome and mapping of the nonstructural proteins NS1, NS2, and NS5. *Virus Genes* 1990;4:197–213.
315. Leonova GN, Isachkova LM, Baranov NI, et al. Investigation of the role of Powassan virus in the etiological structure of tick-borne encephalitis in the Primorskii Ksal. *Vopr Virosol* 1980;2:173–176.
316. Leonova GN, Sorokina MN, Kruglyak SP. Clinical and epidemiological features of Powassan encephalitis in the southern part of the Soviet Far East. *Zh Mikrobiol Epidemiol Immunobiol* 1991;No. 3, pp 35–39.
317. Lepiniec L, Dalgarno L, Huong VT, et al. Geographic distribution and evolution of yellow fever viruses based on direct sequencing of genomic cDNA fragments. *J Gen Virol* 1994;75:417–423.
318. Le Roux JNW. The histopathology of Wesselsbron disease in sheep. *Onderstepoort J Vet Res* 1959;28:237–243.
319. Levine B, Hardwick JM, Trapp BD, et al. Antibody-mediated clearance of alphavirus infection from neurons. *Science* 1991;254:856–860.
320. Levine B, Griffin DE. persistence of viral RNA in mouse brains after recovery from acute alphavirus encephalitis. *J Virol* 1992;66:6429–6435.
321. Lewis JA, Chang GJ, Lanciotti RS, et al. Phylogenetic relationships of dengue-2 viruses. *Virology* 1993;197:216–224.
322. Lin B, Parrish CR, Murray JM, Wright PJ. Localization of a neutralizing epitope on the envelope protein of dengue virus type 2. *Virology* 1994;202:885–890.
323. Littaua RI, Kurane I, Ennis FA. Human IgG Fc RII mediates antibody dependent enhancement of dengue infection. *J Immunol* 1990;144: 3183–3186.
324. Liu Y, Blanden RV, Mullbacher A. Identification of etolytic lymphocytes in West Nile virus-infected murine central nervous system. *J Gen Virol* 1989;70:565–573.
325. Liu Y, King N, Kesson A et al. Flavivirus infection up-regulates the expression of class I and class II major histocompatability antigens on and enhances T cell recognition of astrocytes *in vitro*. *J Neuroimmunol* 1989;21:157–168.
326. Lobigs M, Arthur CR, Müllbacher A, et al. The flavivirus nonstructural protein, NS3 is a dominant source of cytotoxic T cell peptide determinants. 1994;in press.
327. Lobigs M, Dalgarno L, Schlesinger JJ, et al. Location of a neutralization determinant in the E protein of yellow fever virus (17D vaccine strain). *Virology* 1987;161:474–478.
328. Lobigs M, Marshall ID, Weir RC, et al. Murray Valley encephalitis virus field strains from Australia and Papua New Guinea: studies of the sequence of the major envelope protein gene and virulence for mice. *Virology* 1988;165:245–255.
329. Lobigs M, Usha R, Nestorowicz A, et al. Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse virulence. *Virology* 1990;176: 587–595.
330. Lobigs M, Usha R, Nestorowicz A, et al. Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse virulence. *Virology* 1990;176: 587–595.
331. Lobigs M, Weir RC, Dalgarno, L. Genetic analysis of Kunjin virus isolates using Hae III and Taq I restriction digests of single-stranded cDNA to virion RNA. *Austr J Exp Biol Med Sci* 1986;64:185–196.
332. Lopes O, Coimbra TLM, Sacchetta L. de A, et al. Emergence of a new arbovirus disease in Brazil. I. Isolation and characterization of the etiologic agent, Rocio virus. *Am J Epidemiol* 1978;107:444–449.
333. Lopes O, Sacchetta L de A, Coimbra TLM, et al. Emergence of a new arbovirus disease in Brazil. II. Epidemiologic studies on 1975 epidemic. *Am J Epidemiol* 1978;108:394–401.
334. Lopes O, Sacchetta L de A, Nassar E da S, et al. Serological evaluation of a vaccine against human encephalitis caused by Rocio virus. *Rev Inst Med Trop Sao Paulo* 1983;22:108–113.
335. Luby JP, Murphy KF, Grillian JN, Long C-Y, Frank R. Antigenuria in St. Louis encephalitis. *Am J Trop Med Hyg* 1980;29:265–268.
336. Lustig S, Danenberg HD, Kafri Y, Kobiler D, Ben-Nathan, D. Viral neuroinvasion and encephalitis induced by lipoplysaccharide and its mediators. *J Exp Med* 1992;176:707–712.
337. Lyer CGS, Rao RL, Work TH, et al. Kyasanur forest disease. VI. Pathological findings in three fatal human cases of Kyasanur forest disease. *Indian J Med Sci* 1959;13:1011–1022.
338. Mackenzie JS, Smith DW, Broom AK, et al. Australian encephalitis in Western Australia, 1978–1991. *Med J Aust* 1993;158:591–595.
339. Malinosky FJ, Hasty SE, Ussery MA, et al. Prophylactic ribavirin treatment of dengue type 1 infection in rhesus monkeys. *Antiviral Res* 1990; 13:139–150.
340. Malkova D. The role of the lymphatic system in experimental infection with tick-borne encephalitis. I. The tick-borne encephalitis virus in the lymph and blood of experimentally infected sheep. *Acta Virol (Praha)* 1960;4:233.
341. Mandl CW, Guirakoo F, Holzmann H, et al. Antigenic structure of the flavivirus envelope protein E at the molecular level using tick-borne encephalitis as a model. *J Virol* 1989;63:564–571.
342. Mandl CW, Holzmann H, Kunz C, et al. Complete genomic sequence of Powassan virus: Evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. *Virology* 1993;194:173–184.
343. Manuelidas E. Neuropathology of experimental West Nile encephalitis virus infection in monkeys. *J Neuropathol Exp Neurol* 1956,15: 448–456.
344. Marberg K, Goldblum N, Sterk VV, et al. The natural history of West Nile fever. I. Clinical observations during an epidemic in Israel. *Am J Hyg* 1956;64:259–265.
345. Marfin AA, Bleed DM, Lofgren JP et al. Epidemiologic aspects of a St. Louis encephalitis epidemic in Jefferson County, Arkansas, 1991. *Am J Trop Med Hyg* 1993;49:30–37.
346. Marinotti O, James, A., Ribiero J. Diet and salivation in female *Aedes aegypti* mosquitoes. *J Insect Physiol* 1990;36:545–548.
347. Markoff LJ, Innis BL, Houghten R et al. Development of cross-reactive antibodies to plasminogen during the immune response to dengue virus infection. *J Infect Dis* 1991;164:294–301.
348. Marshall ID. Murray Valley and Kunjin encephalitis. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*, vol III. Boca Raton, Fl: CRC Press, 1988;151–190.
349. Mason PW, Dalrymple JM, Gentry MK, et al. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J Gen Virol* 1989;70:2037–2049
350. Mason PW, McAda PC, Dalrymple JM, et al. Expression of Japanese encphalitis virus antigens in *Escherichia coli*. *Virology* 1987;158: 361–372.
351. Mason PW, McAda PC, Mason TL, et al. Sequence of the dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. *Virology* 1987;161:262–267.
352. Mason PW, Pincus S, Fournier MJ, et al. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 1991;180:294–305.
353. Mathews JH, Roehrig JT, Brubaker JR, Hunt AR, Allan JE. A synthetic peptide to the E glycoprotein of Murray valley encephalitis virus defines multiple virus-reactive T- and B-cell epitopes. *J Virol* 1992;66: 6555–6562.
354. Mathews JH, Allan JE, Roehrig JT, et al. T-helper cell and associated antibody response to synthetic peptides of the E glycoprotein of Murray Valley encephalitis virus. *J Virol* 1991;65:5141–5148.
355. Mathiot CC, Georges AJ, Deubel V. Comparative analysis of West Nile virus strains isolated from human and animal hosts using monoclonal antibodies and cDNA restriction digest profiles. *Res Virol* 1990; 141:533–554.

356. Mathur A, Arora KL, Chaturvedi UC. Transplacental Japanese encephalitis virus (JEV) infection in mice during consecutive pregnancies. *J Gen Virol* 1982;59:213–217.

357. Mathur A, Arora KL, Rawat S, et al. Japanese encephalitis virus latency following congenital infection in mice *J Gen Virol* 1986;67: 945–947.

358. Mathur A, Kulshreshta R, Chaturvedi UC. Evidence for latency of japanese encephalitis virus in T lymphocytes. *J Gen Virol* 1989;70: 461–465.

359. Mathur A, Kumar R, Sharma S, et al. Rapid diagnosis of Japanese encephalitis by immunofluorescent examination of cerebrospinal fluid. *Ind J Med Res* 1990;91:1–4.

360. Mayer V. A live vaccine against tick-borne encephalitis: integrated studies. I. Basic properties and behavior of the ES "14" clone (Langat virus). *Acta Virol (Praha)* 1975;19:209–218.

361. McAda PC, Mason PW, Schmaljohn CS, et al. Partial nucleotide sequence of the Japanese encephalitis virus genome. *Virology* 1987;158: 348–360.

362. McIntosh BM, Dickinson DB, McGillivray GM. Ecological studies on Sindbis and West Nile in South Africa. V. Response of birds to inoculation of viruses. *S Afr J Med Sci* 1969;34:77–82.

363. McIntosh BM, Gear JHS. Wesselsbron fever. In: Beran GW, ed. *CRC handbook series in zoonoses, section B: viral zoonoses*, vol 1. Boca Raton, FL, 1981;224–226.

364. McIntosh BM, Jupp PG, Dos Santos I, et al. Epidemics of West Nile and Sindbis viruses in South Africa with *Culex (Culex) univittatus* Theobold as vector. *S Afr J Sci* 1970;72:295–300.

365. McIntosh BM, Kokernot RH, Patterson HE, et al. Isolation of Spondweni virus from four species of culicine mosquitoes and a report of two laboratory infections with the virus. *S Afr Med J* 1961;35: 647–650.

366. McLean DM, Donohue WL. Powassan virus: isolation of virus from a fatal case of encephalitis. *Can Med Assoc J* 1959;80:708–712.

367. McLean RG, Bowen SB. Vertebrate hosts. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980,381–450.

368. Men R, Bray M, Lai CJ. Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice. *J Virol* 1991; 65:1400–1407.

369. Merlo, C, Steffen R, Landis T, Tsai T, Karabatsos N. Possible association of encephalitis and 17D yellow fever vaccination in a 29-year-old traveller. *Vaccine* 1993;11:691.

370. Mitchell CJ, Forattini OP, Miller BR. Vector competence experiments with Rocio virus and three mosquito species from the epidemic zone in Brazil. *Rev Saude Publica* 1986;20:171–177.

371. Miura K, Onodera T, Nishida A, et al. A single gene controls resistance to Japanese encephalitis virus in mice. *Arch Virol* 1990;112:261–270.

372. Mohammed YS, Gressiova M, Adamyova K, et al. Studies on arboviruses in Egypt. II. Contribution of arboviruses to the aetiology of undiagnosed fever among children. *J Hyg (Camb)* 1970;68:491–495.

373. Monath TP. Epidemiology. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980;239–312.

374. Monath TP. Yellow fever. In: Monath TP, ed. *The arboviruses: ecology and epidemiology*. Boca Raton, Florida: CRC Press, 1988; 139–231.

375. Monath TP. Yellow fever: Victor, Victoria? Conqueror, conquest? Epidemics and research in the last forty years and prospects for the future. *Am J Trop Med Hyg* 1991;45:1–43.

376. Monath, TP. Yellow fever and dengue—the interactions of virus, vector and host in the re-emergence of epidemic disease. *Seminars Virol* 1994;5:206.1–206.13.

377. Monath TP, Nasidi A. Should yellow fever vaccine be included in the Expanded Program of Immunization in Africa? A cost-effectivcness analysis for Nigeria. *Am J Trop Med Hyg* 1993;48:274–299.

378. Monath TP. Yellow fever: a medically neglected disease. *Rev Infect Dis* 1987;9:165–175.

379. Monath TP. Pathobiology of the flaviviruses. In: Schlesinger S, Schlesinger MJ, eds. *The Togaviridae and Flaviviridae*. New York: Plenum Press, 1986;375–440.

380. Monath TP, Brinker KR, Chandler FW, et al. Pathophysiologic correlations in a rhesus monkey model of yellow fever. *Am J Trop Med Hyg* 1981;30:431–443.

381. Monath TP, Cetron RB, Adjukiewicz A, et al. Yellow fever in the Gambia, 1978–1979: epidemiologic aspects with observations on the occurrence of Orungo virus infections. *Am J Trop Med Hyg* 1980;29: 912–928.

382. Monath TP, Cropp CB, Bowen GS, Kemp GE, Mitchell CJ, Gardner JJ. Variation in virulence for mice and rhesus monkeys among St. Louis encephalitis virus strains of different origin. *Am J Trop Med Hyg* 1980; 29:948–962.

383. Monath TP, Cropp CB, Harrison AK. Mode of entry of a neurotropic arbovirus into the central nervous system: reinvestigation of an old controversy. *Lab Invest* 1983;48:399–410.

384. Monath TP, Nystrom RR. Detection of yellow fever virus in serum by enzyme immunoassay. *Am J Trop Med Hyg* 1984;33:151–157.

385. Monath TP, Nystrom RR, Bailey RE, et al. Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis.*J Clin Microbiol* 1984;20:784–790.

386. Monath TP, Tsai TF. St. Louis encephalitis: lessons from the last decade. *Am J Trop Med Hyg* 1987;37:40S–59S.

387. Monath TP, Wands JR, Hill LL, et al. Geographic classification of dengue-2 virus strains by antigen signature analysis. *Virology* 1986;154: 313–324.

388. Monath TP. Dengue: The risk to developed and developing countries. *Proc Natl Acad Sci USA* 1994;91:2395–2400.

389. Monath TP, Ballinger ME, Miller BR, et al. Detection of yellow fever viral RNA by nucleic acid hybridization and viral antigen by immunocytochemistry in fixed human liver. *Am J Trop Med Hyg* 1989; 40:663–668.

390. Monlun E, Zeller H, Le Guenо B, et al. Surveillance de la circulation des arbovirus d'interet medical dans la region du Senegal oriental. *Bull Soc Path Exot* 1993;86:21–28.

391. Moore DL, Causey OR, Carey DE, et al. Arthropod-borne viral infections of man in Nigeria, 1964–1970. *Ann Trop Med Parasitol* 1975;69: 49–64.

392. Morens DM, Marchette NJ, Chu MC, Halstead SB. Growth of dengue type 2 virus isolates in human peripheral blood leukocytes correlates with severe and mild dengue disease. *Am J Trop Med Hyg* 1991;45: 644–651.

393. Morens DM, Venkateshan CN, Halstead SB. Dengue 4 virus monoclonal antibodies identify epitopes that mediate immune infection enhancement of dengue 2 viruses. *J Gen Virol* 1987;68:91–98.

394. Morvan J, Besselaar T, Fontenille D, Coulanges P. Antigenic variations in West Nile virus strains isolated in Madagascar since 1978. *Res Virol* 1990;141:667–676.

395. Morvan J, Fontenille D, Lepers J-P, et al. Surveillance serologique de l'infection a virus West Nile dans un village des hauts plateaux a Madagascar. *Ann Soc Belge Med Trop* 1990;70:55–63.

396. Mueller WK, Schaltenbrand G. Attempts to reproduce amyotrophic lateral sclerosis in laboratory animals by inoculation of Sehu virus isolated from a patient with apparent amyotrophic lateral sclerosis. *J Neurol* 1979;220:1–20.

397. Muller D, McDonald M, Stallman N, et al. Kunjin virus encephalomyelitis. *Med J Aust* 1986;144:41–49.

398. Murphy FA. Morphology and morphogenesis. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980;65–104.

399. Myint KS, Nisalak A, Kalayanarooj S, et al. Six hour laboratory confirmation of dengue. Antigen detection in peripheral blood mononuclear cells by immunchistochemistry. *Am J Trop Med Hyg* 1991;45: 173–181.

400. Nasidi A, Monath TP, Vandenberg J, Tomori O, Calisher CH, Hutgen X, Munube GRR, Sorungbe O, Okafor GC, Wali S. Yellow fever vaccination and pregnancy: a four-year prospective study. *Trans Roy Soc Trop Med Hyg* 1993;87:337–339.

401. Nathanson N, Davis M, Thind IS, Price WH. Histological studies of the monkey neurovirulence of group B arboviruses. *Am J Epidemiol* 1966;84:524–531.

402. Nathanson N. Pathogenesis. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980,201–236.

403. Nathanson N, Davis M, Thind IS, et al. Histological studies of the neurovirulence of group B arboviruses. II. Selection of indicator centers. *Am J Epidemiol* 1966;84:524–535.

404. Newton EAC, Reiter P. A model of the transmission of dengue fever with an evaluation of the impact of ultra-low volume (ULV) insecticide applications on dengue epidemics. *Am J Trop Med Hyg* 1992;47: 709–720.

405. Ng, ML, Westaway EG. Phenotypic changes in the flavivirus Kunjin after a single cycle of growth in an *Aedes albopictus* cell line. *J Gen*

Virol 1983;64:1715–1724.
406. Nimmannitya S, Halstead SB, Cohen SN, et al. Dengue and chikungunya virus infections in man in Thailand, 1962–1964. I. Observations on hospitalized patients with hemorrhagic fever. *Am J Trop Med Hyg* 1969;18:954–971.
407. Nisalak A, Halstead SB, Singharaj P, et al. Observations related to pathogenesis of dengue hemorrhagic fever. III. Virologic studies of fatal disease. *Yale J Biol Med* 1970;42:293–310.
408. Nitayaphan S, Grant JA, Chang GJ. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA14-14-2. *Virology* 1990;177:541–552.
409. Nowak T, Wengler G. Analysis of the disulfides present in the membrane proteins of the West Nile flavivirus. *Virology* 1987;156:127–137.
410. Nuttall PA, Jones LD, Labuda M, Kaufman WR. Adaptations of arboviruses to ticks. *J Med Entomol* 1994;31:1–9.
411. Oda K. Antigenic characterization among strains of Japanese encephalitis virus isolated in Hyogo prefecture by the antibody-absorption test. *Kobe J Med Sci* 1976;22:123–137.
412. Ogata A, Nagashima K, Hall WW et al. Japanese encephalitis virus neurotropism is dependent on the degree of neuronal immaturity. *J Virol* 1991;65:880–886.
413. Ogawa M, Okubo H, Tsuji Y, et al. Chronic progressive encephalitis occurring 13 years after Russian spring-summer encephalitis. *J Neurol Sci* 1973;19:363–373.
414. Ohtaki E, Murakami Y, Komori H, Yamashita Y, Matsuishi T. Acute disseminated encephalomyolitis after Japanese B encephalitis vaccination. *Ped Neurol* 1992;8:137–139.
415. Okuno T, Oya A, Ho T. The identification of Negishi virus: a presumably new member of Russian spring-summer encephalitis virus family isolated in Japan. *Jpn J Med Sci Biol* 1961;14:51–59.
416. Okhuysen PC, Crane JK, Pappas J. St. Louis encephalitis in patients with human immunodeficiency virus infection. *Clin Infect Dis* 1993; 17:140–141.
417. Olson JG, Ksiazek TG, Suhandiman et al. 1981 Zika virus, a cause of fever in Central Java, Indonesia. *Trans R Soc Trop Med Hyg* 1981;75: 389–393.
418. Pang T, Devi S, Yeen WP, et al. Lyt phenotype and H-2 compatability requirement of effector cells in the delayed-type hypersensitivity response to dengue virus infection. *Infect Immun* 1984;43:429–431.
419. Pant U, Jacob PG, Bhat HR. Japanese encephalitis virus infection in mosquitoes reared from field collected immatures and in wild-caught males. *Am J Trop Med Hyg* 1989;41:732–736.
420. Panthier R. Epidemiologie du virus West Nile: jaetude d'un foyer en Camargue. I. Introduction. *Ann Inst Pasteur* 1968;114:518–520.
421. Paradas Perez ML, Trujillo Y, Basanta P. Association of dengue hemorrhagic fever with the HLA system. *Hematologia* 1987;20:83–87.
422. Patey O, Ollivaud L, Breuil J, LaFaix C. Unusual neurologic manifestations occurring during dengue fever infection. *Am J Trop Med Hyg* 1993;48:793–802.
423. Paul WS, Mooser PS, Karabatsos N et al. Outbreak of Japanese encephalitis on the island of Saipan, 1990. *J Infect Dis* 1993;167:1053–1058.
424. Pavri KM, Ghalsasi GR, Dastur DK, et al. Dual infections of mice: visceral larva migrans and sublethal Japanese encephalitis virus. *Trans R Soc Trop Med Hyg* 1975;69:99–110.
425. Pavri K. Clinical, clinicopathologic, and hematologic features of Kyasanur Forest disease. *Rev Infect Dis* 1989;(Suppl 4):S854–S859.
426. Peiris JSM, Amerasinghe FP, Amerasinghe PH. Japanese encephalitis in Sri Lanka: I. The study of an epidemic-vector incrimination, porcine infection and human disease. *Trans Roy Soc Trop Med Hyg* 1992;86: 207–323.
427. Perelman A, Stern J. Acute pancreatitis in West Nile fever. *Am J Trop Med Hyg* 1974;23:1150–1152.
428. Pincus S, Mason PW, Konishi E, et al. Recombinant vaccinia virus producing the prM and E proteins of Yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* 1992;187:290–297.
429. Pletnev AG, Bray M, Higgins J, et al. Construction and characterization of chimeric tick-borne encephalitis/dengue type 4 virus. *Proc Natl Acad Sci USA* 1992;89:10532–10536.
430. Pletnev AG, Bray M, Lai CJ. Chimeric tick-borne encephalitis and dengue type 4 viruses: Effects of mutations on neurovirulence in mice. *J Virol* 1993;67:4956–4963.
431. Pogodina VV. The resistance of tick-borne encephalitis virus to the effects of gastric juice. *Vopr Virusol* 1958;3:295–299.
432. Pogodina VV. Experimental study of the pathogenesis of tick-borne encephalitis on alimentary infection. II. Study of pathways of excretion of virus from white mice. *Probl Virol* 1960;5:304–310.
433. Pogodina VV, Frolova MP, Malenko GV, et al. Study on West Nile virus persistence in monkeys. *Arch Virol* 1983;75:71–86.
434. Pogodina VV, Levina LS, Fokina GI, et al. Persistence of tick-borne encephalitis virus in monkeys. III. Phenotypes of the persisting virus. *Acta Virol (Praha)* 1981;25:352–360.
435. Poidinger M, Coelen RJ, Mackenzie JS. Persistent infection of Vero cells by the flavivirus Murray Valley encephalitis virus. *J Gen Virol* 1991;72:573–578.
436. Pool WA, Brownlee A, Wilson DR. The etiology of louping-ill. *J Comp Pathol* 1930;43:253–265.
437. Poland JD, Calisher CH, Monath TP, et al. Persistence of neutralizing antibody to yellow fever virus 30 to 35 years following immunization with 17D yellow fever vaccine: a study of World War II veterans in 1975–1976. *Bull WHO* 1981;59:895–900.
438. Porter KR, Summers PL, Dubois D et al. Detection of West Nile virus by the polymerase chain reaction and analysis of nucleotide sequence variation. *Am J Trop Med Hyg* 1993;48:440–446.
439. Price WH. Chronic disease and virus persistence in mice inoculated with Kyasanur forest disease. *Virology* 1966;29:679–681.
440. Price WH, O'Leary W. Geographical variation in the antigenic character of West Nile virus. *Am J Epidemiol* 1967;85:84–87.
441. Pryor MJ, Wright PJ. The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. *Virology* 1993;194:769–780.
442. Pudney M, Varma MGR. The growth of some tick-borne arboviruses in cell cultures derived from tadpoles of the common frog, *Rana temporaria*. *J Gen Virol* 1971;10:131–138.
443. Puri B, Henchal EA, Burans J, et al. A rapid method for detection and identification of flaviviruses by polymerase chain reaction and nucleic acid hybridization. *Arch Virol* 1994;134:29–37.
444. Putnak R, Feighny R, Burrous J, et al. Dengue 1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicits virus neutralization antibody in mice and protects them from virus challenge. *Am J Trop Med Hyg* 1991;45:159–167.
445. Quick DT, Thompson JM Bond JO. The 1962 epidemic of St. Louis encephalitis in Florida. IV. Clinical features of cases in the Tampa Bay area. *Am J Epidemiol* 1965;81:415–427.
446. Radsel-Medvescek A, Marolt-Gomiscek M, Gajsek-Zima M. Clinical characteristics of patients with TBE treated at the University medical centre hospital for infectious diseases in Ljubljana during the years 1976 and 1977. *Zentralbl Bakteriol* 1980;(Suppl)9:277–280.
447. Radsel-Medvescek A, Marolt-Gomiscek M, Povse-Trojar M, Gajsek-Zima M. Late sequelae after tickborne meningoencephalitis in patients treated at the hospital for infectious diseases university medical centre of Ljubljana during the period 1974–1975. *Zentralbl Bakteriol* 1980; 9(Suppl):281–284.
448. Randolph VB, Hardy JL. Establishment and characterization of St. Louis encephalitis virus persistent infections in *Aedes* and *Culex* mosquito cell lines. *J Gen Virol* 1988;69:2189–2198.
449. Randolph VB, Stollar V. Low pH-induced cell fusion in flavivirus-infected Aedes albopictus cell cultures. *J Gen Virol* 1990;71:1845–1850.
450. Ravi V, Desai AS, Shenoy PK, et al. Persistence of Japanese encephalitis virus in the human nervous system. *J Med Virol* 1993;40:326–329.
451. Reeves WC. *Epidemiology and Control of Mosquito-Borne Arboviruses in California, 1943–1987*. California Mosquito Vector Contr Association, Sacramento, CA 1990.
452. Reid HW. Louping ill. In: Monath TP, ed. *The Arboviruses: epidemiology and ecology*, vol III. Boca Raton, FL: CRC Press, 1988,118–135.
453. Reid HW. Experimental infection of red grouse with louping-ill virus (flavivirus group). *J Comp Pathol* 1975;85:223–230.
454. Reid HW, Doherty PC. Louping-ill encephalomyelitis in the sheep. I. The relationship of viraemia and the antibody response to susceptibility. *J Comp Pathol* 1972;81:521–530.
455. Reid HW, Duncan JS, Phillips JD, et al. Studies on louping-ill virus (Flavivirus group) in wild red grouse (*Lagopus lagopus scoticus*) *J Hyg (Camb)* 1978;81:321–330.
456. Reisen WK, Milby MM, Presser SB, and Hardy JL. Ecology of mosquitoes and St. Louis encephalitis virus in the Los Angeles basin of California, 1987–1990. *J Med Entomol* 1991;29:582–598.
457. Rey F, Harrison S, Heinz FX, et al. X-ray structure of the TBE virus glycoprotein E. *Nature* 1995; (in press).

458. Reyes MG, Gardner JJ, Poland JD, et al. St. Louis encephalitis—quantitative histologic and immunofluorescent studies. *Arch Neurol* 1981; 38:329–334.
459. Reyes, V.M. The pathology of dengue hemorrhagic fever in the Philippines. *Bull WHO* 1966;35:49–50.
460. Rice CM, Grakoui A, Galler R, et al. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *New Biologist* 1989;1:285–296.
461. Rice CM, Lenches EM, Eddy SR, et al. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* 1985;229:726–733.
462. Rice C, Strauss EG, Strauss JH. Structure of the flavivirus genome. In: Schlesinger S, Schlesinger MJ, eds. *The Togaviridae and Flaviviridae.* New York: Plenum Press, 1986;279–326.
463. Rico-Hesse R. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology* 1990;174:479–493.
464. Rivers TM, Schwentker FF. Louping-ill in man. *J Exp Med* 1934;59: 669–678.
465. Robertson BG. Murray Valley encephalitis: pathological aspects. *Med J Aust* 1952;1:107–110.
466. Robertson SE. Yellow Fever. Module 8: the immunological basis for immunization. Geneva: *WHO*, 1993;1–14.
467. Roche JC, Cordellier R, Hervy JP, et al. Isolement de 96 souches de virus dengue 2 a partir de moustiques captures en Cote d'Ivoire et Haute-Volta. *Ann Inst Pasteur Virol* 1983;134E:233–244.
468. Rodhain F, Fetter J-J, Albignac R, Coulanges P, Hannoun C. Arboviruses and lemurs in Madagascar: experimental infection of Lemur fulvus with yellow fever and West Nile viruses. *Am J Trop Med Hyg* 1985;34:816–822.
469. Roehrig JT, Mathews JH, Trent DW. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. *Virology* 1983,128:118–126.
470. Roehrig JT. The use of monoclonal antibodies in studies of the structural proteins of togaviruses and flaviviruses. In: Schlesinger S, Schlesinger MJ, eds. *The Togaviridae and Flaviviridae.* New York: Plenum Press, 1986;251–278.
471. Roehrig JT, Hunt AR, Johnson AJ, et al. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. *Virology* 1989;171:49–60.
472. Roehrig JT, Johnson AJ, Hunt AR, et al. Antibodies to Dengue 2 Jamaica E-glycoprotein synthetic peptides identify antigenic conformation. *Virology* 1990;177:668–675.
473. Roehrig JT, Johnson AJ, Hunt AR, et al. Enhancement of the antibody response to flavivirus B-cell epitopes by using homologous or heterologous T-cell epitopes. *J Virol* 1992;66:3385–3390.
474. Roehrig JT, Risi PA, Brubaker JR, et al. T-helper cell epitopes on the E glycoprotein of dengue 2 Jamaica virus. *Virology* 1994;198:31–38.
475. Roggendorf M, Heinz F, Deinhardt F, et al. Serological diagnosis of acute tick-borne encephalitis by demonstration of antibodies of the IgM class. *J Med Virol* 1981;7:41–50.
476. Rojanasuphot S, Charoensook O-A, Ungchusak, K, et al. A field trial of inactivated mouse brain Japanese encephalitis vaccines produced in Thailand. *Mosq Borne Dis Bull* 1991;8:11–16.
477. Rosen L. Experimental studies of New World monkeys with dengue and yellow fever. *Am J Trop Med Hyg* 1958;7:406–410.
478. Rosen L. The natural history of Japanese encephalitis. *Annu Rev Microbiol* 1986;40:395–414.
479. Rosen L. The pathogenesis of dengue haemorrhagic fever. A critical appraisal of current hypotheses. *S Afr Med J* 1986;11(Suppl):40–42.
480. Rosen L. The pathogenesis of dengue hemorrhagic fever: a critical reappraisal of current hypotheses. *Japan JTrop Med Hyg* 1986;14: 117–122.
481. Rosen L. Sur le mechanism de la transmission verticale du virus de la dengue chez les moustiques. *C R Acad Sci Paris* 1987;304:347–350.
482. Rosen L. Sexual transmission of dengue virus by *Aedes albopictus*. *Am J Trop Med Hyg* 1987;37:398–402.
483. Rosen L, Gubler D. The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg* 1974;23:1153–1160.
484. Rosen L, Lien JC, Shroyer DA, et al. Experimental vertical transmission of Japanese encephalitis virus by *Culex tritaeniorhynchus* and other mosquitoes. *Am J Trop Med Hyg* 1989;40:548–556.
485. Rosenberg S. Neuropathology of Sao Paulo south coast epidemic encephalitis (Rocio encephalitis) *J Neurol Sci* 1980;45:1–12.
486. Rothman AL, Kurane I, Lai C-J, et al. Dengue virus protein recognition by virus-specific murine CD8+ cytotoxic T lymphocytes. *J Virol* 1993;67:801–806.
487. Rudnick A, Marchette NJ, Garcia R. Possible jungle dengue—recent studies and hypotheses. *Apn J Med Sci Biol* 1967;20:69–74.
488. Russell PK, McCown JM. Comparison of dengue 2 and dengue 3 virus strains by neutralization tests and identification of a subtype of dengue 3. *Am J Trop Med Hyg* 1972;21:97–99.
489. Russell PK, Nisalak A. Dengue virus identification by the plaque reduction neutralization test. *J Immunol* 1967;99:291–296.
490. Sabin AB. Nature of inherited resistance to viruses affecting the nervous system. *Proc Natl Acad Sci USA* 1952;38:540–546.
491. Sabin AB. Research on dengue during World War II. *Am J Trop Med Hyg* 1952;1:30–50.
492. Sabin AB, Schlesinger RW. Production of immunity to dengue with virus modified by propagation in mice. *Science* 1945;101:640–642.
493. Sangkawibha N, Rohanasuphot S, Ahandrik S, et al. Risk factors in dengue shock syndrome: A prospective study in Rayong, Thailand. I. The 1980 outbreak. *Am J Epidemiol* 1984;120:653–669.
494. Saluzzo JF, Herve JP, Salaun JJ, et al. Caracteristiques des souches du virus de la fievre jaune isol;aaces des oeufs et des larves d'une tique *Amblyomma variegatum*, recoltes sur le betail a Bangui (Centrafrique). *Ann Virol* 1980;131E:155–165.
495. Sangster MY, Heliams DB, Macjenzie JS, et al. Genetic studies of flavivirus resistance in inbred strains derived from wild mice: evidence for a new resistance allele at the flavivirus resistance locus (Flv). *J Virol* 1993;67:340–347.
496. Santos CD, Post PR, Carvalho R, et al. Complete nucleotide sequence analysis of yellow fever virus vaccine strains 17DD and 17D-213 *Viral Res* 1995; (in press).
497. Scherer WF, et al. Ecological studies of Japanese encephalitis in Japan. Parts I–IX. *Am J Trop Med Hyg* 1959;8:644–722.
498. Scherer WF, Eddy GA, Monath TP, et al. Laboratory safety for arboviruses and certain other viruses of vertebrates. *Am J Trop Med Hyg* 1980;29:1359–1381.
499. Schlesinger RW. *Dengue viruses*. Virology Monograph No. 16. Vienna: Springer-Verlag, 1977.
500. Schlesinger RW. Propagation in chick embryos of the Hawaiian strain of dengue virus. 1. Sustained serial passage in eggs after one hundred and one intracerebral passages in mice. *Am J Hyg* 1950;51:248–254.
501. Schlesinger JJ, Brandriss MW, Monath TP. Monoclonal antibodies distinguish between wild and vaccine strains of yellow fever virus by neutralization, hemagglutination inhibition, and immune precipitation of the virus envelope protein. *Virology* 1983;125:8–17.
502. Schlesinger JJ, Brandriss MW, Cropp CB, et al. Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. *J Virol* 1986;60:1153–1155.
503. Schlesinger JJ, Brandriss MW, Walsh EA. Protection of mice against dengue-2 virus encephalitis by immunization with the dengue-2 virus nonstructural glycoprotein NS-1. *J Gen Virol* 1987;68:853–857.
504. Schlesinger JJ, Brandriss MW, Putnak JR, et al. Cell surface expression of yellow fever virus non-structural glycoprotein NS1: consequences of interaction with antibody. *J Gen Virol* 1990;71:593–599.
505. Schlesinger JJ, Brandriss MW, Walsh EE. Protection against 17 D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp 48 and by active immunization with gp 48. *J Immunol* 1985;135:2805–2809.
506. Schlesinger JJ, Foltzer M, Chapman S. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis inmice. *Virology* 1993;192:132–141.
507. Schlesinger JJ, Putnak JR, Eckels KH. New approaches to flavivirus vaccine development. *Biotechnology* 1992;20:289–307.
508. Schmaljohn C, Blair CD. Persistent infection of cultursed mammalian cells by Japanese encephalitis virus. *J Virol* 1977;24:580–589.
509. Scott RMcN, Eckels KH, Bancroft WH, et al. Dengue 2 vaccine: dose response in volunteers in relation to yellow fever immune status. *J Infect Dis* 1983;148:1055–1060.
510. Scott TW, Lorenz LH, Edman JD. Effects of house sparrow age and arbovirus infection on attraction of mosquitoes. *J Med Entomol* 1990; 27:856–863.
511. Shakar SK, Rao TV, Mruthyunjayanna BP, et al. Autopsy study of brains during an epidemic of Japanese encephalitis in Karnataka, India. *Indian J Med Res* 1983;78:431–440.
512. Sharma S, Mathus A, Prakash V, Kulshreshtha R, Kumar R, Chaturvedi UC. Japanese encephalitis virus latency in peripheral blood lymphocytes and recurrence of infection in children. *Clin Exp Immunol* 1991;85:85–89

513. Shortridge KF, Oya A, Kobayashi M, et al. Arbovirus infections in reptiles: studies on the presence of Japanese encephalitis virus antibody in the plasma of the turtle, *Trionyx sinensis*. *Southeast Asian J Trop Med Public Health* 1975;6:161–169.

514. Shroyer DA. Venereal transmission of St. Louis encephalitis virus by *Culex quinquefasciatus* males (Diptera: Culicidae). *J Med Entomol* 1990;27:334–337.

515. Siler JF, Hall MW, Kitchens AP. Dengue: its history, epidemiology, mechanisms of transmission, etiology, clinical manifestations, immunity and prevention. *Philippine J Sci* 1926;29:1–304.

516. Simmons JS, St. John JH, Reynolds FHK. Experimental studies of dengue. *Philippine J Sci* 1931;44:1–251.

517. Smith A. Genetic resistance to lethal flavivirus encephalitis: effect of host age and immune status and route of inoculation on production of interfering Banzi virus *in vivo*. *Am J Trop Med Hyg* 1981;30:1319–1323.

518. Smith CEG, Varma MGR. Louping-ill. In: Beran GW, ed. *CRC handbook series in zoonoses, section B: viral zoonoses*, vol 1. Boca Raton, FL: CRC Press, 1981;191–200.

519. Smith DW, Broom AK, Keil A, Mackenzie JS. Murray Valley encephalitis soquired in Western Australia. *Med J Austrol* 1991;154: 845–846.

520. Smith TJ, Olson NH, Cheng RH, et al. Structure of human rhinovirus complexed with Fab fragments from a neutralizing antibody. *J Virol* 1993;67:1148–1158.

521. Smithburn KC, Durieux C, Koerber R, et al. Yellow fever vaccination. *WHO Monogr Ser* 1956;30:1–238.

522. Smithburn KC, Paterson HE, Heymann CS, et al. An agent related to Uganda S virus from man and mosquitoes in South Africa. *S Afr Med J* 1959;33:959–962.

523. Smorodintsev AA. Tick-borne spring-summer encephalitis. *Prog Med Virol* 1958;1:210–248.

524. Soper FL. Jungle yellow fever: new epidemiological entity in South America. *Rev Hyg Saude Publica* 1936;10:107–144.

525. Southam CM, Moore AE. West Nile, Ilheus, and Bunyamwera infections in man. *Am J Trop Med* 1951;31:724–741.

526. Southam CM, Moore AE. Induced virus infections in man by the Egypt isolates of West Nile virus. *Am J Trop Med Hyg* 1954;3:19–50.

527. Spence L. St. Louis Encephalitis in tropical America. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980:451–472.

528. Sribougse S, Johnson CM. The first isolation of Bussuquara virus from man. *Trans R Soc Trop Med Hyg* 1971;65:541–542.

529. Srivastava AK, Morita K, Matsuo S, Tanaka M, Igarashi A. Japanese encephalitis virus fusion protein with protein A expressed in Escherichia coli confers protection in mice. *Microbiol Immunol* 1991;35: 863–870.

530. Stephenson JR, Lee JM, Wilton-Smith D. Antigenic variation among members of the tick-borne encephalitis complex. *J Gen Virol* 1984;65: 81–90.

531. Stim TB. Arbovirus plaquing in two simian kidney cell lines. *J Gen Virol* 1969;5:329–338.

532. Stohlman SA, Wisseman CL, Eylar O, et al. Dengue virus induced modifications of host cell membranes. *J Virol* 1975;16:1017–1026.

533. Stokes A, Bauer JH, Hudson JH. Transmission of yellow fever to *Macacus rhesus*, preliminary note. *JAMA* 1928;90:253–254.

534. Stollar V. Studies on the nature of dengue viruses. The structural proteins of type 2 dengue virus. *Virology* 1969;39:426–438.

535. Streatfield R, Bielby G, Sinclair D. A primary dengue 2 epidemic with spontaneous hemorrhagic manifestations. *Lancet* 1993;342:560–561.

536. Strode GK, ed. *Yellow fever*. New York: McGraw-Hill, 1951.

537. Sulkin SE, Allen R. Virus infections in bats. *Monogr Virol* 1974;8:103.

538. Sulkin SE, Burns KF, Shelton DF, Wallis C. Bat salivary gland virus: infections of man and monkey. *Tex Rep Biol Med* 1962;20:113–127.

539. Sumarmo W, Talogo W, Asrin A, et al. Failure of hydrocortisone to affect outcome in dengue shock syndrome. *Pediatrics* 1982;69:45–49.

540. Sumiyoshi H, Mori C, Fuke I, et al. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* 1987;161: 497–510.

541. Sumiyoshi H, Hoke CH, Trent DW. Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. *J Virol* 1992;66:5425–5431.

542. Tan CH-C, Yap E-H, Singh M, et al. Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus. *J Gen Virol* 1990;71:745–748.

543. Taylor RM, Work TH, Hurlbut HS, et al. A study of the ecology of West Nile virus in Egypt. *Am J Trop Med Hyg* 1956;5:579–620.

544. Tesh RB. Experimental studies on the transovarial transmission of Kunjin and San Angelo viruses in mosquitoes. *Am J Trop Med Hyg* 1980; 29:657–666.

545. Theiler M, Anderson CR. The relative resistance of dengue-immune monkeys to yellow fever virus. *Am J Trop Med Hyg* 1975;24:115–117.

546. Theiler M, Smith HH. The use of yellow fever virus modified by in vitro cultivation for human immunization. *J Exp Med* 1937;65:787–800.

547. Thisyakorn U, Nimmannitya S. Nutritional status of children with dengue hemorrhagic fever. *Clin Infect Dis* 1993;16:295–297.

548. Tigertt WD, Berge TO, Gochenour WS, et al. Experimental yellow fever. *Trans NY Acad Sci* 1960;22:323–333.

549. Tiriba A da C, Miziara AM, Lorenco R, et al. Encefalite humana primaria epidemica por arbovirus observada no litoral Sul do Estado de Sao Paulo. *Rev Assoc Med Brasil* 1976;22:415–420.

550. Tomori O, Fagbami A. Fabiyi A. Isolations of West Nile virus from man in Nigeria. *Trans R Soc Trop Med Hyg* 1978;72:103–104.

551. Trent DW, Grant JA, Rosen L, et al. Genetic variation among dengue 2 viruses of different geographic origin. *Virology* 1983;128:271–284.

552. Trent DW, Kinney RM, Johnson BB, et al. Partial nucleotide sequence of St. Louis encephalitis virus RNA: structural proteins, NS1, ns2a, and ns2b. *Virology* 1987;156:293–304.

553. Trent DW, Monath TP, Bowen GS, et al. Variation among strains of St. Louis encephalitis virus: basis for a genetic, pathogenetic, and epidemiologic classification. *Ann NY Acad Sci* 1980;354:219–237.

554. Trent DW, Qureshi AA. Structural and nonstructural proteins of Saint Louis encephalitis virus. *J Virol* 1971;7:379–388.

555. Tsai TF. Arboviral infections in the United States. *Infect Dis Clin N Amer* 1991;5:73–102.

556. Tsai TF. Japanese encephalitis vaccines. In: Plotkin S, ed. *Vaccines*, 2nd ed. Philadelphia: W.B Saunders, 1994;671–713.

557. Tsai TF, Canfield MA, Reed CM, et al. Epidemiological aspects of a St. Louis encephalitis outbreak in Harris County, Texas. *J Infect Dis* 1988;157:351–356.

558. Tsai TF, Cobb WB, Bolin RA, et al. Epidemiologic aspects of a St. Louis encephalitis outbreak in Mesa County, Colorado. *Am J Epidemiol* 1987;126:460–473.

559. Tsai TF, Paul R, Lynberg MC, Letson GW. Congenital yellow fever virus infection after immunization in pregnancy. *J Infect Dis* 1993;168: 1520–1523.

560. Turell MJ. Horizontal and vertical transmission of viruses by insect and tick vectors. In: Monath TP, ed. *The Arboviruses: ecology and epidemiology*, vol I. Boca Raton, FL: CRC Press, 1988;127–152.

561. Turell MJ, Mather TN, Spielman A, et al. Increased dissemination of dengue 2 virus in *Aedes aegypti* associated with concurrent ingestion of microfilariae of *Brugia malayi*. *Am J Trop Med Hyg* 1987;37:197–201.

562. Turell MJ and Spielman A. Nonvascular delivery of Rift valley fever virus by infected mosquitoes. *Am J Trop Med Hyg* 1992;47:190–194.

563. Turell MJ and Tammariello RF. Nonvascular delivery of St. Louis encephalitis and Venezuelan equine encephalitis by infected mosquitoes during feeding on a vertebrate host. *Am J Trop Med Hyg* 1993;49:197.

564. Turell MJ. Virus-dependent mortality in Rift valley fever, eastern equine encephalomyelitis, and chikungunya virus-inoculated mosquito (Diptera: Culicidae) larvae. *J Med Entomol* 1992;29:792–795.

565. Umenai T, Krzysko R, Bektimirov TA, et al. Japanese encephalitis: current worldwide status. *Bull WHO* 1985;63:625–631.

566. Umrigar MD, Pavri KM. Comparative biological studies on Indian strains of West Nile virus isolated from different sources. *Indian J Med Res* 1977;65:596–602.

567. Uren MF, Doherty PC, Allen JE. Flavivirus-specific murine L3T4+ T cell clones: induction, characterization and cross-reactivity. *J Gen Virol* 1987;68:2655–2663.

568. Vasconcelos PF, DaRosa JF, DaRosa AP, et al. Epidcidogia das encefalities por arbovirus na Amazonia brasileira. *Rev Inst Med Trop (Sao Paulo)* 1991;33:465–476.

569. Venugopal K, Buckley A, Reid HW, et al. Nucleotide sequencing of the envelope glycoprotein of Negishi virus shows very close homology to louping ill virus. *Virology* 1992;190:515–521.

570. Vicens R, Robert V, Pignon D, et al. L'epidemie de fievre jaune de l'extreme nord du Cameroun et 1990: premier isolement du virus amaril au cameroun. *Bull WHO* 1993;71:173–176.

571. Vince V, Groevie N. Development of morphological changes in experimental tick-borne meningoencephalitis induced in white mice by different virus doses. *J Neurol Sci* 1969;9:109–130.

572. Vitarana T, de Silva H, Withana N, Gunasekera C. Elevated tumor necrosis factor in dengue fever and dengue hemorrhagic fever. *Caylon*

Med J 1991;36:63–65.
573. Vorndam AV. Immunization. In: Monath TP, ed. *St. Louis encephalitis*. Washington, DC: APHA, 1980:623–636.
574. Walker PJ, Henchal EA, Blok J, et al. Variation in dengue type 2 viruses isolated in Bangkok during 1980. *J Gen Virol* 1988;69:591–602.
575. Wang G, Porta C, Chen Z, et al. Identification of a Fab interaction footprint site on an icosahedral virus by cryoelectron microscopy and X-ray crystallography. *Nature* 1992;355:275–278.
576. Waters TD, Anderson PS Jr, Beebe GW, et al. Yellow fever vaccination, avian leukosis virus, and cancer risk in man. *Science* 1972;177: 76–77.
577. Watts DM, Burke DS, Harrison BH. Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue 2 virus. *Am J Trop Med* 1987;36:143–152.
578. Webb HE, Chatterjee JB. Clinicopathological observations in monkeys infected with Kyasanur forest disease virus, with special reference to the haematopoietic system. *Br J Haematol* 1962;8:401–413.
579. Webb HE, Connolly JH, Kane FF, et al. Laboratory infections with louping-ill with associated encephalitis. *Lancet* 1968;2:255.
580. Webb, HE, Rao RL. Kyasanur forest disease: a general clinical study in which some cases with neurological complications were observed. *Trans R Soc Trop Med Hyg* 1961;55:284–298.
581. Webster LT. Inherited and acquired factors in resistance to infection: development of resistance and susceptible lines of mice through selective breeding. *J Exp Med* 1933;57:793–817.
582. Weiss KE, Haig DA, Alexander RA. Wesselsbron virus—a virus not previously described, associated with abortion in domestic animals. *Onderstepoort J Vet Res* 1956;27:183–195.
583. Wengler G, Wengler G. Cell-associated West Nile flavivirus is covered with E + Pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J Virol* 1989; 63:2521–2526.
584. Wengler G, Wengler G. An analysis of the antibody response against West Nile virus E protein purified by SDS-PAGE indicates that this protein does not contain sequential epitopes for efficient induction of neutralizing antibodies. *J Gen Virol* 1989;70:987–992.
585. Wengler G, Wengler G. The NS3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* 1993;197: 265–273.
586. Westaway EG. Assessment and application of a cell line from pig kidney for plaque assay and neutralization tests with twelve group B arboviruses. *Am J Epidemiol* 1966;84:439–456.
587. Westaway EG, Brinton MA, Gaidamovich SY, et al. Flaviviridae. *Intervirology* 1985;24:183–192.
588. Wheelock EF, Edelman R. Specific role of each human leukocyte type in viral infections. III. 17D yellow fever virus replication and interferon production in homogeneous leukocyte cultures treated with phytohemagglutinin. *J Immunol* 1969;103:429–436.
589. Whitby JE, Jennigs AD, Barrett ADT. Nucleotide sequence of the envelope protein gene of the tick-borne flavivirus, Kumlinge A 52. *Virus Genes* 1993;7:145–149.
590. White MG, Carter NW, Rector FC, et al. Pathophysiology of epidemic St. Louis encephalitis. I. Inappropriate secretion of antidiuretic hormone. *Ann Intern Med* 1969;71:691–702.
591. Whitehead RH, Chaicumpa V, Olson LC, et al. Sequential dengue virus infections in the white-handed gibbon (*Hylobates lar*). *Am J Trop Med Hyg* 1970;19:94–102.
592. Wiemers MA, Stallman ND. Immunoglobulin M in Murray Valley encephalitis. *Pathology* 1975;7:187–192.
593. Wills MR, Singh BK, Debnath NC, Barrett ADT. Immunogenicity of wild-type and vaccine strains of Japanese encephalitis virus and the effect of haplotype restriction on murine immune responses. *Vaccine* (in press).
594. Winkler G, Heinz FX, Kunz C. Characterization of a disulfide bridge-stabilized antigenic domain of tick-borne encephalitis virus structural glycoprotein. *J Gen Virol* 1987;68:2239–2244.
595. Winkler G, Randolph VB, Cleaves GR, et al. Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology* 1988;162:187–196.
596. Wisseman CL Jr, Sweet RH, Rosenzweig EC, et al. Attenuated living type 1 dengue vaccines. *Am J Trop Med Hyg* 1963;12:620–623.
597. Wolfe MS, Calisher CH, McGuire K. Spondweni virus infection in a foreign resident of Upper Volta. *Lancet* 1982;2:1306–1308.
598. Woodall JP, Roz A. Experimental milk-borne transmission of Powassan virus in the goat. *Am J Trop Med Hyg* 1977;26:190–192.
599. Work TH, Rodriguez FR, Bhatt PN. Kyasanur forest disease: virological epidemiology of the 1958 epidemic. *Am J Public Health* 1959;49: 869–874.
600. World Health Organization. *Technical information on Japanese encephalitis and guidelines for treatment.* New Delhi: WHO Regional Office Southeast Asia, 1979.
601. World Health Organization. *Technical guide for diagnosis, treatment, surveillance, prevention, and control of dengue haemorrhagic fever.* Geneva: WHO, 1982.
602. Yoksan S, Bhamarapravati N, Halstead SB. Dengue virus vaccine development: Study on biological markers of uncloned dengue 1–4 viruses serially passaged in primary kidney cells. In: St Georg TD, Kay BH, Blok J, eds. *Arbovirus research in Australia*. Brisbane: Proceedings of the Fourth Symposium CSIRO/QIMR, 1986;35–38.
603. Yoshida I, Takagi M, Inokuma E, et al. Establishment of an attenuated ML-17 strain of Japanese encephalitis. *Biken J* 1981;24:47–67.
604. Young P, Bletchly C, Mackenzie J. Antigenic and structural analysis of the Dengue virus glycoprotein, NS1. In: Uren MF, ed. *Arbovirus research in Australia*. Brisbane: Queensl Inst Med Res, 1993;138–142.
605. Young PR, Falconar AKI. Nonstructural proteins as virus vaccines. In: Uren MF, ed. *Arbovirus research in Australia* Brisbane: Queensl Inst Med Res, 1993;62–67.
606. Yu YX, Zhang GM, Guo YP, et al. Safety of a live-attenuated Japanese encephalitis virus vaccine (SA-14-14-2) for children. *Amer J Trop Med Hyg* 1988;39:214–217.
607. Zhang P-F, Klutch M, Muller J, Marcus-Sekura CJ. Susceptibility of the Sf9 insect cell line to infection with adventitious viruses. *Biologicals* 1994;22:205–213.
608. Zhang Y-M, Hayes EP, McCarty TC, et al. Immunization of mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis. *J Virol* 1988;62:3027–3031.
609. Zilber LA. Pathogenicity of Far Eastern and Western (European) tick-borne encephalitis viruses in sheep and monkeys. In: Libikova H, ed. *Biology of viruses of the tick-borne encephalitis complex*. New York: Academic Press, 1960;260–265.
610. Zilber LA, Soloviev VD. Far eastern tick-borne spring-summer encephalitis. *Annu Rev Sov Med Spec* 1946;5(Suppl):1–75.
611. Zivny J, Kurane I, Tacket CO, Edelman R, Ennis FA. Dengue virus-specific, human CD4+ cytotoxic T lymphocytes generated in short term culture. *Viral Immunol* 1993;6:143–151.
612. Zlotnik I, Carter GB, Grant DP. The persistence of louping-ill virus in immunosuppressed guinea pigs. *Br J Exp Pathol* 1971;52:395–403.
613. Zlotnik I, Grant DP, Carter GB. Experimental infection of monkeys with viruses of the tick-borne encephalitis complex: degenerative cerebellar lesions following inapparent forms by the disease or recovery from clinical encephalitis. *Br J Exp Pathol* 1976;57:200–210.

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CHAPTER 32

Flaviviridae: The Viruses and Their Replication

Brett D. Lindenbach and Charles M. Rice

At the dawn of human virus research, nearly one century ago, Walter Reed demonstrated that the disease known as yellow fever could be experimentally transferred via the filtered serum of an infected individual (565) and that this infectious agent was transmitted to man by mosquitoes (566). The discovery of this viral pathogen eventually led to the derivation of a live-attenuated strain that has been effectively used for human vaccination for over 60 years (452,664,701). It is now appreciated that yellow fever virus (YF) is but one representative of a large family of related positive-strand RNA viruses, the *Flaviviridae* (from the Latin *flavus*, yellow). This family currently consists of three genera: the flaviviruses, the pestiviruses (from the Latin *pestis*, plague), and the hepaciviruses (from the Greek *hepar*, *hepatos*, liver) (177,761). In addition to these genera, a group of unassigned viruses, the GB agents, are awaiting formal classification within the family. As detailed later, members of this family exhibit diverse biologic properties and a lack of serologic cross-reactivity, although they share similarities in virion morphology, genome organization, and presumed RNA replication strategy. The increasing significance of *Flaviviridae* as human and animal pathogens (Chapters 33 and 34) emphasizes that their study remains no less pertinent than in Reed's time.

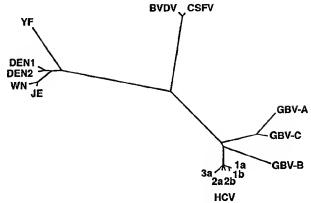


FIG. 1. The *Flaviviridae*. Phylogenetic tree based on parsimony analysis of NS3 helicase regions. Shown are members of the *Flavivirus* genus: yellow fever virus (*YF*), dengue-1 (*DEN-1*), dengue-2 (*DEN-2*), West Nile virus (*WN*), and Japanese encephalitis (*JE*); the *Pestivirus* genus: bovine viral diarrhea virus (*BVDV*) and classical swine fever (*CSFV*); several hepacivirus (*HCV*) isolates; and the unclassified viruses GBV-A, GBV-B, and GBV-C. (Figure adapted from ref. 640, with permission.)

The flaviviruses and pestiviruses were formerly considered to be members of the family *Togaviridae*, based on traditional methods of virus classification. However, through the study of viral genes, virus structure, and the viral replication cycle, it became apparent that these viruses represented an evolutionarily distinct group, and they have been subsequently reclassified as a separate virus family, the *Flaviviridae* (761). The diversity of viruses within the *Flaviviridae* is partially illustrated in Figure 1 and Table 1. Comparative sequence analysis has permitted classification of positive-stranded RNA viruses based on inferred evolutionary relationships. The *Flaviviridae* are members of the positive-strand virus supergroup 2, bearing distant similarity in their RNA-dependent RNA polymerases (RdRps) to coliphages and the plant-infecting carmo-, tombus-, diantho-, and subgroup I luteoviruses (345). The *Flaviviridae* are the only member of polymerase supergroup 2 to also encode RNA helicases, although members of polymerase supergroup 1 contain similar helicases. Thus this family of viruses is likely to exhibit a unique mode of genome replication.

FAMILY CHARACTERISTICS AND REPLICATION CYCLE

Common features believed to be shared by the three genera and highlights of the replication cycle are diagrammed in Figure 2. Our understanding of these steps is far from complete and the current view is based largely on studies with flaviviruses. Modifications are likely as more information becomes available, particularly for hepatitis C virus (HCV) and the pestiviruses. Enveloped virions are composed of a lipid bilayer with two or more species of envelope (E) proteins surrounding a nucleocapsid that consists of a single-stranded positive-sense genome RNA complexed with multiple copies of a small, basic capsid (C) protein.

Binding and uptake are believed to involve receptor-mediated endocytosis via cellular receptors specific for viral envelope proteins. Fusion of the virion envelope with cellular membranes delivers the nucleocapsid to the cytoplasm, where translation of the genome RNA occurs. The organization of the genome RNA is similar for all genera. All known viral proteins are produced as part of a single long polyprotein of more than 3,000 amino acids that is cleaved by a combination of host and viral proteases. The structural proteins are encoded in the N-terminal portion of the polyprotein, with the nonstructural proteins in the remainder. Sequence motifs characteristic of a serine protease, RNA helicase, and an RdRp are found in similar locations in the polyproteins of all three genera (444). The cleavage products containing these regions are believed to form the enzymatic components of the RNA replicase. RNA replication occurs in cytoplasmic replication complexes that are associated with perinuclear membranes, and it occurs via synthesis of a genome-length minus-strand RNA intermediate. RNA synthesis is resistant to actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Progeny virions are thought to assemble by budding through intracellular membranes into cytoplasmic vesicles. These vesicles follow the host secretory pathway, fuse with the plasma membrane, and release mature virions into the extracellular compartment.

THE FLAVIVIRUSES

Background and Classification

The flavivirus genus consists of nearly 80 viruses, many of which are arthropod-borne human pathogens. Flaviviruses cause a variety of diseases including fevers, encephalitis, and hemorrhagic fevers (Chapter 33). Entities of major global concern include dengue fever with its associated dengue hemorrhagic fever (DHF) and shock syndrome (DSS) (reviewed in refs. 219, 234, and 584), Japanese encephalitis (451), and yellow fever (reviewed in ref. 709). Other flaviviruses of regional or endemic concern include Kyasanur Forest disease, Murray Valley encephalitis (MVE), St. Louis encephalitis, tick-borne encephalitis (TBE), and West Nile (WN) viruses. Decreases in mosquito control efforts during the latter part of the 20th century, coupled with societal factors such as increased transportation and dense urbanization, have contributed to the reemergence of flaviviruses such as dengue (DEN) in South and Central America (453,

TABLE 1. *Members of the* Flaviviridae

Flaviviruses Antigenic Group (#,[a] vector[b])	Type members
Tick-borne encephalitis (12, T)	Central European encephalitis (TBE-W) Far Eastern encephalitis (TBE-FE)
Rio Bravo (6, T[c])	Rio Bravo
Japanese encephalitis (10, M)	Japanese encephalitis (JE) Kunjin (KUN) Murray Valley encephalitis (MVE) St. Louis encephalitis (SLE) West Nile (WN)
Tyuleniy (3, T)	Tyuleniy
Ntaya (5, M[c])	Ntaya
Uganda S (4, M)	Uganda S
Dengue (4, M)	Dengue type 1 (DEN-1) Dengue type 2 (DEN-2) Dengue type 3 (DEN-3) Dengue type 4 (DEN-4)
Modoc (5, U)	Modoc
Ungrouped (17, M[c])	Yellow fever (YF)

Pestiviruses Species	Type member
Bovine viral diarrhea virus 1 (BVDV-1)	BVDV strain NADL
Bovine viral diarrhea virus 2 (BVDV-2)	BVDV strain 890
Hog cholera or classical swine fever virus (CSFV[d])	CSFV Alfort/187
Border disease virus (BDV)	BDV BD31

Hepaciviruses Species	Type member
Hepatitis C virus (HCV)[e]	HCV-1

Unassigned[f] Group	Type member
GB virus-A-like viruses	GB virus-A (GBV-A)
GB virus-B	GB virus-B (GBV-B)
GB virus-C	GB virus-C (GBV-C, HGV[g])

[a]Number of recognized members in each antigenic group (from ref. 86, with permission).

[b]Arthropod vectors: T, tick; M, mosquito; U, unidentified or no vector.

[c]Arthropod vectors for some members of these groups have not been identified. The ungrouped flaviviruses include mosquito- and tick-transmitted viruses as well as some with no known vector.

[d]In the pestivirus literature, HCV has been a common abbreviation for hog cholera virus. More recent publications and this chapter use CSFV to avoid confusion with the human hepatitis C viruses.

[e]The hepatitis C viruses include a large number of isolates, which can be divided into six major genotypes and over 100 subtypes on the basis of genetic divergence (for example, see refs. 81, 582, 636).

[f]Several animal and human viruses most closely related to HCV have recently been described (see ref. 638 for review). These viruses have been tentatively assigned to the *Flavivirdae* based on their genomic organization and genetic similarity to recognized members of the family.

[g]GBV-C and hepatitis G virus (HGV) refer to the same viral agent (386, 640). Currently, it is unclear if this prevalent human virus is associated with clinical disease.

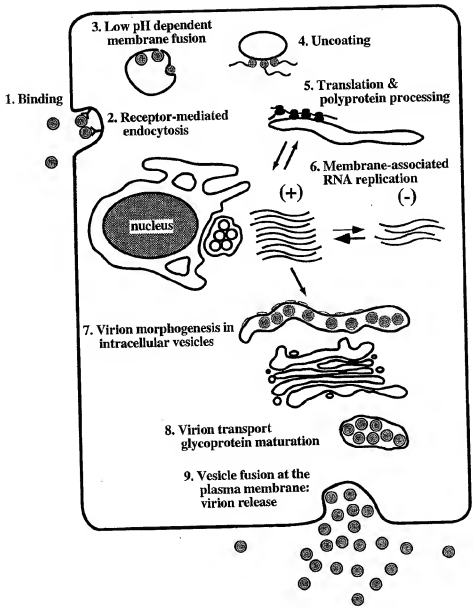


FIG. 2. Life cycle of the *Flaviviridae*. See the text for further details.

480). In 1999, WN virus was isolated for the first time in the western hemisphere during an outbreak in New York City that was responsible for several human cases of encephalitis, including four deaths (17,22,358).

Vaccination is available for YF, using the live-attenuated 17D strain, and for TBE and Japanese encephalitis (JE) using inactivated virus (253). Efforts to derive live-attenuated strains of other flaviviruses have met with only limited success. Development of effective DEN vaccines that exhibit cross-protection, thought to be important for preventing subsequent dengue-associated immunopathogenesis (see later), are proving to be particularly challenging. The ability to genetically manipulate flaviviruses, described later, is being used to develop novel approaches to vaccine design (65,95,223,224,408,526). One promising candidate live-attenuated vaccine is a chimeric flavivirus created by replacing the structural glycoproteins of YF-17D with those of JE (95,224,454, 455). A similar approach to create a vaccine against DEN-2 is under investigation (223).

Viruses within the genus are categorized into antigenic complexes and subcomplexes based on classical serologic criteria (85,86) or into clusters, clades, and species, according to molecular phylogenetics (349,807). These latter methods have permitted the classification of viruses such as YF, which lacks close relatives. Mosquito-

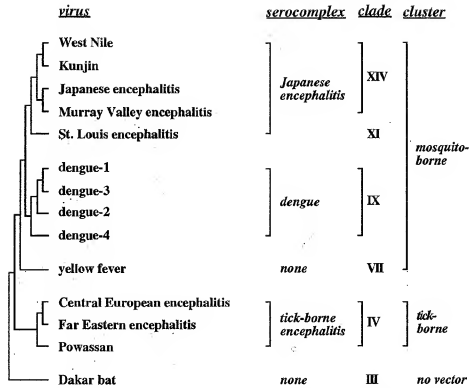


FIG. 3. Organization of the *Flavivirus* genus. The dendrogram on the *left* shows the relationships of selected flaviviruses based on a recent phylogenetic analysis (349). Evolutionary distance is not represented in this figure. The serologic and phylogenetic classifications of these viruses are indicated to the *right*.

borne and tick-borne flaviviruses, although quite distinct, appear to have evolved via a common ancestral line that diverged from non-vector-borne viruses (i.e., those for which no arthropod vectors are known) (349,807). The salient features of flavivirus taxonomy are illustrated in Figure 3, with emphasis given to viruses that are mentioned in this chapter. Two additional points should be clarified with regard to this genus organization. First, the trivial name tick-borne encephalitis virus is commonly applied to both Central European encephalitis virus and Far Eastern encephalitis virus, although they clearly represent distinct viruses with differences in vector species, geographical distribution, and sequence relatedness (153). In addition, because of the high mutation rate of RNA viruses, it is expected that each flavivirus exists as a complex, interrelated population of quasi-species (Chapter 13). Recent evidence for intertypic recombination among DEN viruses hints at the unrealized diversity potentially lurking within flavivirus populations (774).

Experimental Systems

Flaviviruses can be cultured in whole animals such as chick embryos, suckling mouse brain, and mosquitoes, as well as in primary or established cell lines of mammalian, avian, or insect origin. In vertebrate cells, dramatic cytopathic and ultrastructural effects can occur, including vacuolation and proliferation of intracellular membranes (472); infection is commonly cytocidal, although some vertebrate cell types do not show these effects and become chronically infected. Even during the peak of virus production, a major inhibition of host macromolecular synthesis is not observed (68,713,759,760). Arthropod cells in culture may demonstrate cytopathic effects, most frequently manifested as cell fusion and syncytia formation (reviewed in refs. 68, 663, and 712). However, infection of mosquito cells is often noncytopathic, and persistent infections can be established (476). Mosquitoes remain chronically infected for life and produce extremely high levels of infectious virus particles in the salivary glands.

The life cycle of flaviviruses is increasingly understood at the molecular genetic level, and complete genome sequences exist for at least one member of each taxonomic group (see, for example, refs. 232, 248, 322, 412, and 573). Furthermore, flaviviruses can be recovered from cells containing RNA transcripts of viral cDNAs, allowing flavivirus biology to be probed with reverse genetics (213,218,281,310,322,331,354,366,410, 527,572,665). Such reverse genetic systems are also

being exploited to express foreign genes (323,726), to provide stable genetic stocks for live-attenuated flavivirus vaccines (331,572), and to assist in the design of novel vaccine strategies (65,95,223,224,408,526).

Structure and Physical Properties of the Virion

Flavivirus particles appear to be spherical, 40 to 60 nm in diameter, containing an electron dense core (about 30 nm diameter) surrounded by a lipid bilayer (Fig. 4A) (472). Mature virions sediment between 170 and 210S, have a buoyant density of 1.19 to 1.23 g/mL, and are composed of 6% RNA, 66% protein, 9% carbohydrate, and 17% lipid (592,712). Because of the lipid envelope, flaviviruses are readily inactivated by organic solvents and detergents (592). Three viral proteins are associated with virions: the E (envelope), M (membrane), and C (capsid) proteins. The E protein is the major surface protein of the viral particle, probably interacts with viral receptors, and mediates virus–cell membrane fusion. Antibodies that neutralize virus infectivity usually recognize this protein (reviewed in ref. 253), and mutations in E can affect virulence (reviewed in Chapter 33). M protein is a small proteolytic fragment of prM protein, which is important for maturation of the virus into an infectious form, as described later. Discrete nucleocapsids, composed of C protein and genomic RNA (120 to 140S, buoyant density 1.30 to 1.31 g/mL), can be isolated after solubilization of the envelope with nonionic detergents (592).

Binding and Entry

It is thought that flaviviruses attach to the surface of host cells through an interaction of E protein with one or more receptors, and many E-reactive antibodies have been shown to neutralize virus infectivity by interfering with virus binding (251,280). The patterning of receptor expression in animal tissues is likely to contribute to flavivirus tropism *in vivo*, although such receptors have not been specifically identified. Several cell surface proteins have been described as candidate flavivirus receptors (47,329,346,416,470,548,596). In addition, recombinant E protein from DEN-2 virus has been shown to interact with highly sulfated glycosaminoglycans, and cell surface expression of heparan sulfate was required for efficient infection of mammalian cells by a laboratory-passaged strain of DEN-2 virus (107). Consistent with a role for glycosaminoglycans in flavivirus entry, the infectivity of DEN viruses can be partially neutralized by incubation with heparin or highly sul-

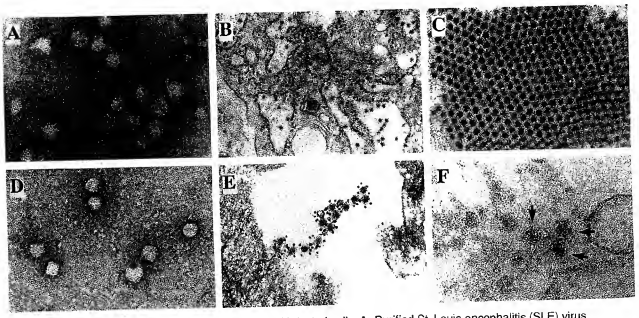


FIG. 4. Electron micrographs of virions and infected cells. **A:** Purified St. Louis encephalitis (SLE) virus negatively stained with ammonium molybdate (472). Surface projections appear as a very thin, indistinct layer. (Courtesy of Dr. Frederick A. Murphy.) **B:** Thin section of a BHK-21 cell at 48 hours after infection showing SLE virions in the cisternae of the endoplasmic reticulum (765). (Courtesy of Frederick A. Murphy, Sylvia G. Whitfield, and A. K. Harrison.) **C:** Para-crystalline array of SLE virus in a *Culex pipiens* mosquito salivary gland cell 25 days after blood-meal-feeding on an infected suckling mouse. (Courtesy of Sylvia G. Whitfield, Frederick A. Murphy, and W. Daniel Sudia.) **D:** Classical swine fever virus (CSFV) virions negatively stained with uranyl acetate. (Courtesy of Dr. Frank Weiland.) **E:** Ultrathin section of STE cells infected with CSFV and immunostained with E^{rns}-specific monoclonal antibody (MAb) 24/16 and colloidal gold. Bar = 100 nm. (From ref. 746, with permission.) **F:** Thin section showing virus-like particles (*arrows*) in HPBALL cells harvested 14 days after infection with HCV (632). Particles measured approximately 50 nm in diameter. (Courtesy of Dr. Yokho Shimizu.)

fated heparan (107,280). An additional mechanism of flavivirus binding, typically referred to as antibody-dependent enhancement (ADE), has been demonstrated for a number of flaviviruses cultured *in vitro* (236–238,388,509–511,520,616). ADE involves the increased binding of virus opsinized with subneutralizing concentrations of flavivirus-reactive antibodies to cells expressing immunoglobulin Fc receptors (509,616). It has been hypothesized that this mechanism might occur *in vivo* and, by altering tissue tropism, could contribute to the enhanced pathogenesis frequently observed in secondary infections by other DEN types (reviewed in refs. 234, 235, and 584).

After binding, it is generally believed that virions are taken up by receptor-mediated endocytosis (192,292,481, 487), although direct fusion at the plasma membrane has also been observed (244,245). Ultrastructural studies have localized single virions and virion aggregates to clathrin-coated pits on the cell surface, and uptake of virus particles into coated vesicles rapidly follows attachment (192,194). Virions are later found in uncoated prelysosomal vesicles, where an acid-catalyzed membrane fusion is thought to release the nucleocapsid into the cytoplasm (192,194,254,481). Consistent with this, a conformational change in the viral E protein, which probably exposes a fusogenic domain (567; and see later), occurs at low pH (11,221,254,330,583,611,659). Acid pH can promote fusion of virions with liposomal membranes *in vitro* or at the plasma membrane of cultured cells (193,194,222,330,550,667), although in the latter case this mode of entry does not lead to productive infection (194,330). Following entry and fusion, nucleocapsids are presumably disassembled, genomic RNA is translated, and RNA is initiated.

Genome Structure

The genome of flaviviruses consists of a single-stranded RNA about 11 kilobases (kb) in length. This RNA contains a 5′ cap ($m^7G5'ppp5'A$) at the 5′ end and lacks a polyadenylate tail (756). Genomic RNA is the messenger RNA for translation of a single long open reading frame (ORF) as a large polyprotein, as described later. Surrounding the ORF are 5′ and 3′ noncoding regions (NCRs) of around 100 nucleotides (nt) and 400 to 700 nt, respectively. These regions contain conserved sequences and predicted RNA structures that are likely to serve as cis-acting elements directing the processes of genome amplification, translation, or packaging. Although it is not yet feasible to directly study the structures of flavivirus RNAs *in vivo*, the ability of RNA to adapt alternative foldings could regulate these competing processes (231,538).

The 5′ NCR sequence is poorly conserved between flaviviruses, but it appears to contain common secondary structural elements that influence the translation of flavivirus genomes (69,84,231). However, the most significant function of the 5′ NCR probably resides in its reverse complement, the 3′ NCR of viral minus strands, which forms the site for initiation of plus-strand synthesis. Deletions engineered into the 5′ NCR of DEN-4 were shown to have dramatic effects on the ability to recover live viruses, but they do not correlate with the translational efficiency of the mutant genomes (84). Interestingly, one of the resultant mutants exhibited a limited host-range growth phenotype, suggesting potential interactions of this region (or its reverse complement) with host-specific factors. In keeping with this, cellular proteins were shown to bind specifically to a terminal stem-loop in the 3′ NCR of WN virus minus strand (627).

The 3′ NCR of the flavivirus genome, which presumably functions as a promoter for minus-strand synthesis, exhibits a great deal of sequence divergence and size heterogeneity. Nevertheless, computational analyses have revealed features that are common to all flaviviruses or that are conserved within specific taxonomic groups, and that tend to cluster in a region proximal to the 3′ end (538,552,735,739). RNA folding predictions based on thermodynamic considerations, covariation analyses, and biochemical probing support the existence of a 90- to 120-nt stem-loop structure at the 3′ terminus of all flavivirus genomes, with the potential to form a pseudoknot involving an adjacent stem-loop (70,206,231,411,412, 538,552,573,627,666,677,751,811). Several lines of evidence suggest a functional role for this structure in viral RNA replication. Mutations engineered within the 3′ terminal stem-loop of the genome of DEN-2 virus greatly affected the ability to recover live viruses, and one of the recovered viruses exhibited a host restricted-growth phenotype (808). Several cellular proteins bind to this region of WN virus RNA, including the phosphorylated form of translation elongation factor 1α (49,50). Furthermore, viral replicase proteins NS3 and NS5 have been shown to bind to the terminal stem-loop RNA *in vitro* (103,133). The conserved portion of the 3′ NCR also contains consensus sequences (CS1 and CS2) that are retained among all mosquito-borne flaviviruses, one of which has the potential to base-pair with a conserved region in the 5′ region of the ORF, suggesting that cyclization of flavivirus genomes is possible (231). A DEN-4 genome engineered to contain a deletion in CS1 indicates that this element is required for virus viability (429). Complementarity in the proposed cyclization sequences was recently shown to be required for efficient RdRP activity *in vitro*, although the upstream element could be supplied in trans (803). Similarly, stretches of conserved RNA sequence and potential cyclization sequences are also found in the genome RNAs of tick-borne flaviviruses (412,413,415). Other regions in the 3′ NCR, which may be dispensable for virus replication in culture, nevertheless appear to be important determinants for growth in mammalian hosts, and further analysis of such regions is likely to contribute greater insight into flavivirus pathogenesis and vaccine development (414,429,537).

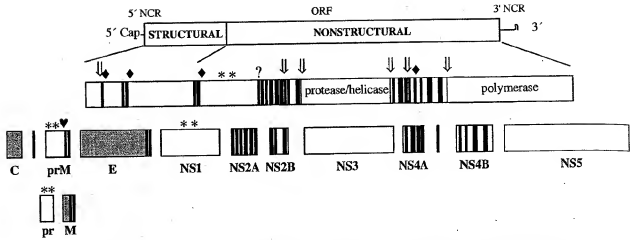


FIG. 5. Translation and processing of the flavivirus polyprotein. At the top is depicted the viral genome with the structural and nonstructural protein coding regions, the 5′ cap, and the 5′ and 3′ NCRs indicated. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Mature structural proteins are indicated by *shaded* boxes and the nonstructural proteins and structural protein precursors by *open boxes*. Contiguous stretches of uncharged amino acids are shown by *black bars*. *Asterisks* denote proteins with N-linked glycans but do not necessarily indicate the position or number of sites utilized. Cleavage sites for host signalase (♦), the viral serine protease (⇩), furin or other Golgi-localized protease (♥), or unknown proteases (?) are indicated.

Translation and Proteolytic Processing

The flavivirus genome is translated as a large polyprotein that is processed co- and posttranslationally by cellular proteases and a virally encoded serine protease (described later) into at least 10 discrete products. As depicted in Figure 5, the N-terminal one quarter of the polyprotein encodes the structural proteins, and the remainder contains the nonstructural (NS) proteins, in the following order: C-prM-E-NS1-NS2A-NS3-NS4A-NS4B-NS5 (573). Based on the deduced hydrophobicity of the viral proteins, their predicted membrane topology is illustrated in Figure 6. This model is supported by experimental data (89,757), although the transmembrane regions have not been well defined.

Features of the Structural Proteins

The C Protein

C protein (about 11 kd) is highly basic (55,573,711), consistent with its proposed role in forming a ribonucleoprotein complex with packaged genomic RNA. Basic residues are concentrated at the N- and C-termini of C, and they probably act cooperatively to specifically bind genomic RNA (323). The central portion of C contains a hydrophobic domain that interacts with cellular membranes and may play a role in virion assembly (418). The nascent C protein (anchC) contains a C-terminal hydrophobic domain (491) that acts as a signal sequence for translocation of prM into the lumen of the endoplas-

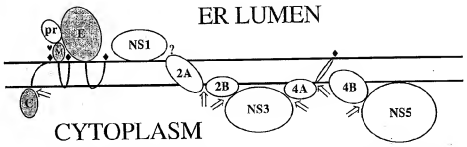


FIG. 6. Membrane topology of flavivirus proteins. The proposed orientation of the flavivirus polyprotein cleavage products with respect to the ER membrane is shown. The proteins are drawn to scale (areas are proportional to the number of amino acids) and arranged in order (left to right) of their appearance in the polyprotein. Mature structural proteins are *shaded* and C-terminal membrane-spanning segments of M and E are indicated. Cleavage sites for host signalase (♦), the viral serine protease (⇩), furin or other Golgi-localized protease (♥), or unknown proteases (?) are indicated.

mic reticulum (ER). Mature C protein is generated by viral serine protease cleavage at a site upstream of this hydrophobic domain (16,395,785).

The prM Protein

The N-terminus of prM (about 26 kd) is generated in the ER by host signal peptidase. This processing event appears to require prior processing of anchC by the cytoplasmic viral serine protease (16,395,788), although the temporal order of these cleavages has been questioned (787,788). However, in support of this model, signalase cleavage at this site can be made viral protease-independent by cleaving anchC with another protease (660) or by improving the context of the signalase cleavage site (366,661). Interestingly, improvement of the prM signalase cleavage context was shown to be lethal for YF virion production (366). Thus, the availability of viral protease activity may regulate structural protein processing, and therefore virion assembly, over the course of an infection. During the egress of virions through the secretory pathway, prM is cleaved by the trans-Golgi resident enzyme furin (653), to form the structural protein M (about 8 kd) and the N-terminal "pr" segment, which is secreted into the extracellular medium (474). The N-terminal "pr" segment is predominantly hydrophilic and contains one to three N-linked glycosylation sites (93) and six conserved cysteine residues, all of which participate in intramolecular disulfide bridges (492). The structural protein M, located in the C-terminal portion of prM, is present in mature virions and contains a shortened ectodomain (about 41 amino acids) followed by two potential membrane-spanning domains. Antibodies to prM can mediate protective immunity (316), perhaps by neutralization of released virions that contain some uncleaved prM (see later).

The E Protein

E protein (about 50 kd) is a type I membrane protein, containing adjacent transmembrane domains in the C-terminus that serve to anchor this protein to the membrane and as the signal sequence for NS1 translocation (573). E protein contains 12 highly conserved Cys residues that form intramolecular disulfide bonds (492), and it is often glycosylated (93,768).

The three-dimensional structure of TBE E protein was determined by x-ray crystallography of a soluble tryptic fragment obtained from purified virions (Fig. 7) (567). These data indicate that E protein is divided into three structural domains and forms head-to-tail homodimeric rods that are thought to lie parallel to the virion surface in

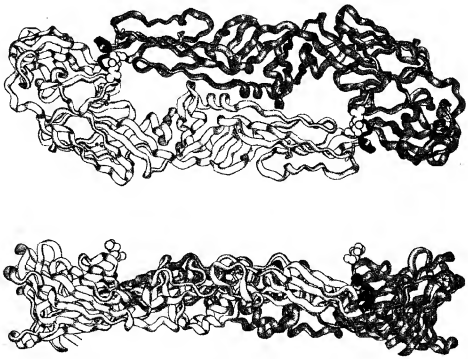


FIG. 7. Envelope protein structure. The structure of a tick-borne encephalitis (TBE) E glycoprotein dimer is represented in this ribbon diagram, as viewed perpendicularly (*top*) or laterally (*bottom*) with respect to the lipid bilayer. Individual E monomers are colored *gray* or *white*, and the proposed fusogenic domains are in *black*. (Kindly provided by Steve Allison and F. X. Heinz. Adapted from ref. 567, with permission.)

a meshlike network. This structural model provides a framework for understanding the molecular basis of E protein interactions. Structural regions of the ectodomain contain putative receptor binding sites (107,567), a potential fusogenic domain (567), contact sites for E homodimer formation (567), a region involved in prM–E interaction (12), and the trigger for low-pH-induced rearrangement (659). E protein homodimers disassociate at low pH, and each monomer reassociates with two adjacent E proteins, perhaps around a three-fold axis of symmetry at the stalk, to form trimeric complexes (11,254, 659). E trimers are thought to extend outward from the virion surface, presumably exposing the hydrophobic fusogenic domains. The stem-anchor region of E, for which structure was not determined, contains determinants for E trimer formation and for stabilization of prM-E heterodimers (12,741). Mutations in the putative receptor binding site in the TBE E protein gave rise to viruses with attenuated growth in culture and reduced virulence in mice (409). For some mosquito-borne flaviviruses, a similar region of E protein contains a putative integrin-binding motif Arg-Gly-Asp. Mutation of this sequence in E of MVE virus produced viruses with decreased entry kinetics, increased reliance on glycosaminoglycans for entry, and attenuated virulence (366).

Features of the Nonstructural Proteins

The NS1 Protein

The NS1 glycoprotein (M_r of about 46 kd) exists in cell-associated, cell-surface, or extracellular nonvirion forms. NS1 is translocated into the ER lumen and released from the C-terminus of E by signal peptidase (161,571,757). It contains 12 highly conserved cysteines that form intramolecular disulfide bonds, and it is rapidly glycosylated on two or three Asn residues (367,419,646). NS1 is cleaved from its downstream neighbor, NS2A, around 10 minutes after synthesis by an unknown, membrane-bound, ER-resident host protease (93,160,161). The eight C-terminal hydrophobic residues of NS1, and more than 140 amino acids of NS2A, are necessary determinants of this cleavage (93,160,272,517). Truncated and elongated forms of NS1, which presumably contain alternate C-terminal cleavage sites, have also been described for some viruses (53,419). In addition, murine cells persistently infected with JE virus have been found to produce truncated forms of NS1, although this appears to be caused by an uncharacterized adaptation of the host cells rather than by alterations within the viral genome (104). Twenty to 40 minutes after synthesis, NS1 forms homodimers that are resistant to denaturation with 6M urea or 5M guanidinium-HCl, but that are unstable at high temperatures or low pH (769). Dimers of NS1 exhibit a partially hydrophobic nature, pelleting with membranes and fractionating equally into the aqueous and detergent phases upon extraction with Triton X-114 (769). A point mutation has been identified in Kunjin NS1 that destabilizes NS1 dimers and confers a replication defect on the virus (233), indicating a functional role for NS1 dimerization. NS1 appears to be peripherally associated with membranes, as it does not contain any putative transmembrane domains or known lipid modifications, and it can be released from membranes by sodium carbonate (pH 11.5), 8M urea, or 5M guanidinium-HCl (161,757).

NS1 is slowly secreted from mammalian cells and is not secreted from mosquito cells (419,530,770). During secretion, one of the N-linked glycans is modified to contain complex sugars (367,419,477,540), and three NS1 dimers come together into a soluble hexameric form (132,170).

NS1 was first characterized as the soluble (non-virion-associated) complement-fixing (SCF) antigen present in the sera and tissues of experimentally infected animals (63). It is now understood that the extracellular forms of NS1 strongly elicit humoral immune responses, and immunization with purified or recombinant NS1 can be protective (159,297,383,546,617,706). Furthermore, protective immunity can be passively transferred with antibodies against NS1, apparently by their ability to direct complement-mediated lysis of infected cells via interaction with the cell-surface-associated form of NS1 (255, 618,619). The secretion of a viral NS protein that elicits protective immune responses is one of the more curious aspects of flavivirus biology that await further inquiry.

Several lines of evidence implicate NS1 in the process of RNA replication. NS1 has been found to co-localize with markers of RNA replication in association with membrane structures that are presumed sites of replication (404,764). Mutations at the first or both N-linked glycosylation sites of NS1 dramatically impair virus replication (477,539) and demonstrate a decrease in viral RNA accumulation (477). Furthermore, a YF mutant containing a temperature-sensitive lesion in NS1 showed a profound decrease in RNA accumulation under nonpermissive conditions (478). NS1 can be supplied in trans to a YF genome lacking a functional NS1 gene, and RNA replication is blocked at a very early stage in the absence of complementing NS1 (384). Further genetic analysis has revealed that DEN NS1 does not productively interact with the YF replicase in trans (385). This block in replication can be suppressed by a mutation in NS4A, indicating that an interaction between NS1 and NS4A is critical for replicase function and suggesting a potential mechanism whereby NS1 participates in the cytoplasmic process of RNA replication (385).

The NS2A and NS2B Proteins

NS2A is a relatively small (about 22 kd), hydrophobic protein of unknown function. Cleavage of NS1-2A occurs

in the ER, as previously mentioned, whereas the C-terminus is generated via cleavage at the NS2A/2B junction by the cytoplasmic serine protease, indicating that this protein must be membrane spanning. An alternative cleavage within YF NS2A can also be utilized by the viral protease, leading to a C-terminally truncated form of this protein that is about 2 kd smaller in mass (92,483). Mutations that block cleavage at either site are lethal for some aspect of YF replication (483). NS2A has been localized to presumed sites of RNA replication, and *in vitro* studies with a recombinant glutathione-S-transferase-KUN NS2A fusion protein suggest that this protein binds to NS3 and NS5, as well as to RNA transcripts of the 3′ NCR (405). Thus, this protein might function in recruitment of RNA templates to the membrane-bound replicase.

NS2B is a small (about 14 kd) membrane-associated protein containing two hydrophobic domains surrounding a conserved hydrophilic region (573,757). It forms a complex with NS3 and is a required cofactor for the serine protease function of NS3 (20,90,94,162,299). Deletion analysis indicates that a 40-amino-acid region in the central conserved domain of NS2B is required for NS2B-3 protease activity, and mutations in the central conserved domain that destabilize interaction with NS3 abolish protease activity (94,121,146,162,299).

The NS3 Protein

NS3 is a large (about 70 kd) cytoplasmic protein that associates with membranes via its interaction with NS2B (20,94,121). NS3 contains several enzymatic activities that implicate this protein in polyprotein processing and RNA replication.

The N-terminal one third of NS3 contains homology to trypsin-like serine proteases (34,35,198), an enzymatic activity that has been confirmed by deletion analysis (97,163,534,752) and by site-directed mutagenesis of the residues comprising the proposed catalytic triad (YF NS3 residues His-53, Asp-77, and Ser-138) (97,541,722,752, 810) or the substrate-binding pocket (533,722). Mutations in NS3 that abolish protease activity also prevent the recovery of viable mutant viruses, substantiating a crucial role for this enzyme in the virus life cycle (94). Expression of the N-terminal 167 to 181 residues of NS3, together with NS2B, is sufficient to form the active protease (90,373). The NS2B-3 protease cleaves in both cis and trans configurations, and it mediates cleavages at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions, as well as cleavages that generate the C-termini of mature C (16,786) and NS4A (377), and minor cleavages within NS2A (483) and NS3 (20,700). Alignment of known cleavage sites and mutation of these residues have been used to characterize the substrate specificity of this enzyme. These data indicate that the NS2B-3 protease preferentially cleaves after pairs of basic residues and before an amino acid with a small side chain (94,96, 378,483). However, some nonconservative mutations at these sites are tolerated, and several canonical dibasic cleavage motifs within the polyprotein are apparently not utilized, suggesting that contextual information contributes to cleavage site selection. The structure of a recombinant DEN-2 NS3 protease domain, in the absence of cofactor NS2B, has recently been resolved to 2.1 Å by using x-ray crystallography (475). This model exhibits structural similarities to other serine proteases including the NS3-4A protease of HCV (described later), provides a framework for the interpretation of NS3 mutagenesis studies, and suggests that NS2B could contribute to substrate binding specificity (475).

The C-terminal three quarters of NS3, which slightly overlaps the serine protease domain (373), has been implicated in RNA replication. This region has significant homology to RNA helicases containing the motif Asp-Glu-Ala-(Asp/His) (199). These enzymes utilize the energy of nucleoside triphosphate (NTP) hydrolysis to power RNA unwinding (302). RNA-stimulated NTPase activity has been demonstrated for full-length and N-terminally truncated NS3 (373,744,754). Recently, DEN-2 NS3 has been shown to contain an NTP-dependent RNA unwinding activity, consistent with the function of NS3 as an RNA helicase (373). The precise role of an RNA helicase is not known for flaviviruses (302), but it may help to dissociate nascent RNA strands from their template during RNA replication, or perhaps unwind secondary structures involved in template recognition or initiation of RNA synthesis. In this regard, NS3 was shown to bind to a region of the 3′ NCR containing the terminal stem-loop, in association with NS5 (103,133). A minor truncated form of NS3 has been described that apparently results from recognition of a cryptic NS2B-3 protease cleavage site within the helicase domain of NS3 (20, 541,700). The biologic consequences of this alternative processing event are not well understood, although it may serve to disrupt the helicase function of NS3. In addition to the NTPase/RNA helicase activity of NS3, the C-terminal region of this protein also contains an RNA triphosphatase activity, which is distinct from the NTPase activity (755). This enzyme is likely to be involved in modifying the 5′ end of the genome in preparation for 5′ cap addition by a guanylyltransferase activity.

NS4A and NS4B

NS4A and NS4B are relatively small (about 16 kd and 27 kd, respectively) hydrophobic proteins that are membrane associated (377,405,762). Based on its subcellular distribution (405) and interaction with NS1 (385), NS4A appears to function in RNA replication, perhaps by anchoring replicase components to cellular membranes. NS4B also localizes to presumed sites of RNA replication, but it also appears to be dispersed throughout cytoplasmic membranes, and possibly the nucleus (762).

The N-terminus of NS4A is generated by the NS2B-3 protease (394), whereas the C-terminus of NS4A contains a transmembrane domain that serves to translocate NS4B into the ER. The NS2B-3 protease cleaves at a site within NS4A, just upstream of this signal sequence, and mutations that block this cleavage also block subsequent signal peptidase cleavage in the ER lumen to generate the N-terminus of NS4B (377,533). This unusual coordinated processing scheme, which is strikingly similar to processing at the C–prM junction, might serve to regulate replicase function. Furthermore, unprocessed NS3-4A and NS4A-4B have been detected in flavivirus-infected cells (93,394,533), suggesting that polyprotein cleavage in this region may be inefficient or controlled in additional ways. NS4B is posttranslationally modified to a form that appears to be about 2 kd smaller than the nascent protein, although the nature of this modification is unknown (93,533).

NS5

NS5, the largest (about 103 kd) and most conserved flavivirus protein, contains sequence homology to RdRPs of other positive-strand RNA viruses, including the invariant Gly-Asp-Asp (GDD) motif common to these enzymes (573). Purified recombinant NS5 exhibits RdRP activity in primer extension reactions (681), and NS5 protein has been found to fractionate with RdRP activity in infected cell extracts (116). NS5 also shares homology with methyltransferase enzymes involved in RNA cap formation and is thus probably involved in methylation of the 5′ RNA cap structure (344). Site-directed mutagenesis has confirmed that motifs implicated in polymerase and methyltransferase activities are essential for virus replication, and that the functions of NS5 can be supplied in trans (318–321). NS5 can be phosphorylated by an unknown cellular Ser/Thr kinase (311,466,561), a feature that is also conserved in the NS5A proteins of the pestiviruses and HCVs (see later). The role of NS5 phosphorylation remains obscure, but it may regulate the interaction of NS5 and NS3 (311), or a subcellular redistribution of NS5 into the cell nucleus (176,311).

RNA Replication

Following translation and processing of the viral proteins, a viral replicase is assembled from NS proteins, the viral RNA, and presumably some host factors. The replicase associates with membranes, probably through interactions involving the small hydrophobic NS proteins. Replication begins with the synthesis of a genome-length minus-strand RNA, which then serves as a template for the synthesis of additional plus-strand RNAs. The first round of minus-strand accumulation has been detected just over 3 hours after infection (384). Viral RNA synthesis appears to be asymmetric *in vivo*, with a plus-strand accumulation more than 10 times greater than that of minus strands (120,477). Viral minus strands appear to accumulate even late after infection and have been isolated exclusively in double-stranded forms (120,756). Flavivirus replication can be followed *in vivo* by metabolic labeling of virus-specific RNAs in the presence of actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Three major species of labeled flavivirus RNAs have been described, one type sedimenting at 40S, another at 20S, and a heterogeneous population at 20S to 28S (120,756). 40S RNA is sensitive to RNase treatment and is identical to virion-associated RNA, consistent with its being genomic RNA (756). 20S RNA, frequently termed the replicative form (RF), is likely to be a transiently stable duplex of viral plus- and minus-strand RNAs based on RNase resistance and conversion to an RNase sensitive form that comigrates with 40S RNA by heat denaturation (756). The 20S to 28S RNAs are partially sensitive to RNase treatment and are described as replicative intermediate (RI) RNAs that most likely contain duplex regions and recently synthesized plus-strand RNAs displaced by nascent strands undergoing elongation (117,120). Pulse-chase analyses indicate that RF and RI RNAs are precursors to the 40S (genomic) RNA (117,120). This mode of replication, with minus strands serving as templates for the production of multiple plus strands, can be described as semiconservative and asymmetric (117).

RdRP activity has been studied *in vitro* by using crude cytoplasmic preparations of infected cells, and similar RNA forms have been demonstrated in these reactions (31,115,117,214–216,803). However, these *in vitro* systems appear to involve chain elongation rather than *de novo* initiation, and the complete process of flavivirus RNA replication has not yet been fully reconstructed in an *in vitro* model. Physical characterization of these preparations indicates that flavivirus RdRP activity cosediments with dense membrane fractions enriched for several NS proteins and membrane structures morphologically similar to those found in virus-infected cells (116). RdRP activity has also been retained after solubilization in various detergents (116,214), although an intact flavivirus replicase has not yet been purified.

Membrane Reorganization and the Compartmentalization of Flavivirus Replication

Several studies have described ultrastructural changes in membranes of flavivirus-infected cells, predominately in the perinuclear region. In general, the earliest event is the proliferation of ER membranes (473,486), followed by the appearance of smooth membrane structures around the time of early logarithmic virus production. Smooth membrane structures are small clusters of vesicles containing electron-dense material within the lumen of smooth ER (87,363,473,486,488). These structures

continue to accumulate during later times of infection, when they become adjacent to newly formed convoluted membranes. Convoluted membranes appear to be contiguous with the ER as randomly folded membranes or highly ordered structures that are sometimes described as paracrystalline arrays (363,473). All of these membrane alterations are concentrated in densely sedimenting membrane fractions from Kunjin-infected cells that are enriched for RdRP activity along with viral proteins NS2A, NS2B, NS3, NS4A, NS4B, and possibly NS1 (116). With the improved preservation of cellular membranes afforded by cryosectioning, Mackenzie et al. (404) demonstrated the appearance of vesicle packets, clusters of vesicles (each 100 to 200 nm in diameter) bound by a smooth membrane, during late times of infection. It seems likely that vesicle packets are related to or identical with smooth membrane structures, and they are often associated with smooth ER or Golgi-like membranes undergoing a morphologic process of wrapping. Such structures are also enriched for markers of the trans-Golgi (403).

The subcellular sites of RNA replication have been probed by metabolic labeling of nascent RNAs (64,662,763), by immunolabeling with sera reactive to double-stranded RNA, which presumably recognizes RF and RI RNAs (404,405,763,764), and by *in situ* hybridization (212). Apart from one report of nuclear replication (64), all investigations concur that viral RNAs accumulate in association with cytoplasmic membranes in the perinuclear region of mammalian cells and, in particular, with vesicle packets (404). NS1, NS2A, NS3, and NS4A have all been shown to localize to vesicle packets (404,405,764). Thus, it appears that along with double-stranded RNA, proteins implicated in RNA replication associate with vesicle packets. In contrast, the components of the viral serine protease, NS2B and NS3, colocalize with convoluted membranes (764). The membrane reorganization that occurs in infected cells might therefore give rise to adjacent, but distinct subcellular structures where viral polyprotein processing or RNA replication take place. It should however be emphasized that vesicle packets have been described only at late times after infection compared to the onset of RNA replication, and the sites of early RNA synthesis have not been defined. Furthermore, much remains to be learned about how interactions among NS proteins (103,133,311,385, 405), between NS proteins and viral RNA (103,133,405), and between viral RNA and host factors (49,50,627) combine to form an active replicase.

Assembly and Release of Virus Particles

Ultrastructural studies indicate that virion morphogenesis occurs in association with intracellular membranes (reviewed in ref. 472). Electron microscopic studies of flavivirus-infected cells have consistently demonstrated morphologically mature virions within the lumen of a compartment believed to be the ER (142,246,247,292, 333,363,404,424,473,485,497,650,651,740). In many studies, virions appear to accumulate within disorderly arrays of membrane-bound vesicles (see Fig. 4B). Budding intermediates and clearly distinguishable cytoplasmic nucleocapsids have not been frequently observed, suggesting that the process of assembly is rapid. Nascent virions are believed to be transported by bulk flow through the secretory pathway to the cell surface, where exocytosis occurs. Budding of virions at the plasma membrane has been occasionally observed (246,424,497, 650), and it does not appear to be a major mechanism for virion formation. These ultrastructural observations, together with studies on structural protein biosynthesis, oligomer formation, and the properties of intracellular and released virions, suggest the following model for virion assembly and maturation: The highly basic C protein interacts with viral genomic RNA in the cytoplasm to form a nucleocapsid precursor. The orientation of C, prM, and E with respect to the ER membrane would suggest that nucleocapsids acquire an envelope by budding into the ER lumen. Cosynthesis of E and prM is necessary for proper folding of E (341). These proteins have been shown to be associated as detergent-stable heterodimers (11,12,254,741,753) that can form higher-order structures, which may represent virion-associated lattices (741).

Later stages in virion maturation include glycan modification of E (for some viruses) and prM by trimming and terminal addition (93,419,491), implying that virions move through an exocytosis pathway similar to that used for synthesis of host plasma membrane glycoproteins. Although differences in the efficiency of prM cleavage have been noted (474,741), this cleavage generally distinguishes released virus from intracellular virus particles (626). Intracellular M-containing virions have not been detected, suggesting that prM cleavage occurs just prior to release of mature virions. This cleavage can be inhibited by elevating the pH in intracellular compartments (254,550,626), consistent with catalysis by furin (254, 550). Although inhibition of prM cleavage does not impair virus release, studies on prM-containing particles suggest that this cleavage is required to generate highly infectious virus (11,220,254,551,626,653,753). As seen for the alphavirus structural proteins (729–731, and see Chapter 29), experimental data suggest that flaviviruses use oligomerization and prM cleavage to regulate the activation of E-protein-mediated fusion activity. The current hypothesis is that the function of prM in the prM–E heterodimer is to prevent E from undergoing an acid-catalyzed conformational change during transit of immature virions through an acidic intracellular compartment (220,222,254,550,753). Upon cleavage of prM and release of mature virus, the E–M interaction is destabilized (753). The differences in intracellular and extracel-

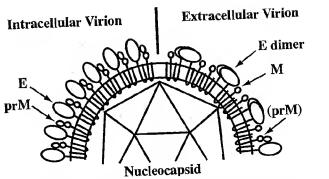


FIG. 8. Envelope proteins of intracellular and extracellular flavivirus virions. (Adapted from ref. 91, with permission.)

lular virions are illustrated in Figure 8. The hemagglutination activity exhibited by flaviviruses, which depends on low pH, probably results from activation of the fusogenic activity of E protein.

In addition to mature virions, slowly sedimenting (70S) noninfectious particles, which are also capable of agglutinating red blood cells at acid pH (called SHA, for slowly sedimenting hemagglutinin; for review, see ref. 592), are released from flavivirus-infected cells. SHA particles appear as 14-nm doughnut-like structures and are composed of E and M, with variable amounts of prM (592). Recent studies have shown that expression of prM and E is sufficient for release of SHA-like particles (11,341,420). These particles, which are fusogenic, provide an excellent model system for examining the functions of E and prM (12,611), and they show promise as immunogens that elicit protective immunity (128,342, 343,420,524).

Generation of Defective Flaviviruses and the Involvement of Host Resistance Genes

Defective-interfering (DI) particles have been valuable tools for the study of RNA virus replication, and DIs can contribute to viral pathogenesis *in vivo*. For some virus families, strongly interfering DI particles, containing truncated and rearranged genomic RNAs, are easily generated by high-multiplicity passage. These RNAs contain cis-acting sequences necessary for replication and packaging but do not encode a complete or functional set of viral proteins, and therefore they need a helper virus to supply these functions in trans. In the case of flaviviruses, although DI particles have been observed in persistently infected vertebrate cell cultures, strongly interfering DIs are not readily obtained under these conditions or during serial high-multiplicity passaging (68). Several potential explanations exist for this observation, including the possibility that, under the conditions tested, most of the virus-specified components of the replication machinery are required in cis. In this regard, some viral replicase components can be supplied in trans (151,266,318–321, 384), whereas several Kunjin virus nonstructural genes or their gene products are apparently required in cis (321). It is unclear whether this latter observation is the result of defects at the protein or RNA level. One group has recently described DI genomes in Vero cells persistently infected with MVE virus (357). Characterization of these RNA species indicated that they contain in-frame deletions of 2.3 to 2.6 kb in the prM, E, and NS1 genes. It is unlikely that the truncated NS1 is functional, and it is most likely complemented in trans, although it may contribute to interference. Of the few flavivirus DIs that have been characterized, all contain in-frame deletions, suggesting that selective pressure maintains the downstream ORF for the translation of one or more NS proteins in cis.

A system in which DIs appear to be readily generated has been studied in some detail (reviewed in ref. 68). Investigations into the heritable susceptibility of mice to YF infection (608) led to the discovery that multiple dominant alleles at a single locus on chromosome 5 can confer resistance to flaviviruses (67,598–601,719,720). Flaviviruses can replicate in resistant mice, but the spread of infection is slower, with significantly lower peak viremias (10^3- to 10^4-fold) than in susceptible mice. In primary fibroblasts from resistant mice, viral RNA synthesis was reduced, lower titers of infectious virions were released, and a high proportion of DIs were found even after a single growth cycle (67). Analyses of viral RNAs in the brains of infected mice suggest a block in RNA replication but do not necessarily support the existence of DI particles in this system (721). Nevertheless, these results indicate that a specific, but as yet unidentified, host gene can have dramatic effects on flavivirus RNA synthesis. Interestingly, RNA–protein complexes involving the 3′ NCR of the viral minus strand were found to be less stable in cell extracts prepared from a flavivirus-resistant mouse strain (627).

THE HEPATITIS C VIRUSES

Background and Classification

Following the development of diagnostic tests for hepatitis A and B viruses, an additional agent, which could be experimentally transmitted to chimpanzees (14,265,671), became recognized as the major cause of transfusion-acquired hepatitis. The causative agent, previously designated non-A, non-B hepatitis, and now referred to as hepatitis C virus, was identified in 1989 through expression cloning of cDNAs derived from the serum of an experimentally infected animal (113). Recognition of this pathogen led to the development of diagnostic tests for screening blood supplies, which dramatically decreased the incidence of posttransfusion hepatitis. Nevertheless, about 170 million people, roughly 3% of the human population, are infected with

HCV (19), and virus transmission remains a significant public health concern. The most notable feature of HCV infections is that they typically persist, often lasting for decades, with more than 70% of cases progressing to chronic hepatitis, a condition that predisposes patients to developing chronic active hepatitis, cirrhosis of the liver, and hepatocellular carcinoma (Chapter 34). In addition to the sequelae resulting from chronic liver infection, HCV has been associated with other human diseases. Of nearly 30 such associations, which include a number of autoimmune diseases, the linkage between HCV infection and mixed type II cryoglobulinemia is the most striking. This disease is characterized by symptoms ranging from palpable purpura and fatigue, to life-threatening vasculitis of vital organs, such as the kidneys (2). Treatment for HCV typically involves the use of interferon-α, with or without ribavirin, although poor response to these treatments is not uncommon. The considerable diversity among HCV isolates, the emergence of genetic variants in chronically infected individuals, and the low level of protective immunity elicited after HCV infection present major challenges to the development of more effective therapies and vaccines. HCV is extremely refractile to growth in culture and there are currently no good animal models of this disease outside the chimpanzee. Despite these obstacles, intense scientific scrutiny over the past decade has yielded an astonishing wealth of information about this virus and its components. Much has been learned about the enzymology of viral replication proteins, and high-resolution crystallographic analyses have provided structural information for approximately 40% of the HCV genome and more than half of the nonstructural region. Understanding the biologic roles of these proteins is likely to come from recent progress in efficient initiation of HCV RNA replication in culture.

HCV is the sole member of the *Hepacivirus* genus (582). Hepaciviruses share some common features with the pestiviruses including genome organization, limited sequence relatedness, and a similar mechanism of translational control. HCV exhibits even greater similarity to the recently described GB agents, but there are also some important differences such as the potential to encode an obvious capsid protein. Within the *Hepacivirus* genus, numerous isolates of HCV are currently grouped into six major genotypes and several subtypes based on phylogenetic analysis of complete genome sequences or subgenomic fragments (81,636), although this taxonomic scheme appears to be inadequate for fully describing the diversity of recent HCV isolates, particularly those from Southeast Asia (582). It remains unclear whether there is a correlation between HCV genotype and disease severity or clinical outcome, although patients with genotype 1 viruses are clearly less responsive to treatment with antivirals (see ref. 457 and references therein).

Experimental Systems

Progress toward an understanding of HCV molecular biology has been hampered by its limited replication in cell culture and the lack of small animal models. Most of the early work, aimed at defining the physical properties of the virus and pathogenesis induced during acute and chronic infection, involved clinical samples from patients with posttransfusion hepatitis and chronic liver disease, or from experimental infection of chimpanzees (734). In chimpanzees, HCV RNA is detected in the serum as early as 3 days after inoculation and typically increases to 10^5 to 10^7 HCV RNA molecules per milliliter during the acute phase. Little evidence of hepatocellular damage is seen despite these high levels of viremia. Two to 3 months after infection, inflammatory infiltrates are seen in the liver, with areas of focal necrosis and release of enzymes such as alanine amino transferase (ALT) into circulation. This acute hepatitis coincides with the emergence of HCV-specific cellular immune responses and the appearance of HCV-specific antibodies in many (but not all) cases. In humans, acute hepatitis with overt clinical disease is seen in only about 25% of those infected. Circulating virus becomes undetectable and is apparently spontaneously cleared in 20% to 30% of cases (15). In chimpanzees, the rate of acute resolved infection may be somewhat higher (32). Chronic infections predominate, with only rare instances of clearance without treatment with interferon alone or in combination with ribavirin. Although HCV was originally thought to replicate poorly *in vivo*, recent models based on measuring viral loads after interferon therapy (356,484) or plasmapheresis (549) suggest a production rate of about 10^{12} virions per day and a virion half-life of 2 to 3 hours.

Since 1997, the availability of functional cDNA clones and infectious transcribed RNAs have allowed studies in the chimpanzee model to define the importance of viral replication determinants and to follow the evolution of the virus and host immune responses in acute-resolving versus chronic infections. For example, it has been shown that the HCV-encoded enzymes and conserved RNA elements in the 3′ NCR are essential (340,792) and that extensive variation in the glycoproteins or elsewhere are not required to establish chronic infection (406). Subunit vaccination studies (112) have provided evidence for protective immunity and encourage efforts to develop HCV vaccines for health care workers and other high-risk groups.

In contrast to the chimpanzee model, efforts to develop small-animal models for HCV infection have met with only limited success (202). One model, an irradiated beige/nude/X-linked immunodeficient (BNX) mouse reconstituted with bone marrow from mice with severe combined immunodeficiency disease (SCID), and engrafted with human liver tissue, supported limited HBV and HCV replication (189). Similarly, immunodefi-

cient mice engrafted with human hepatocytes under the kidney capsule and maintained long term by stimulation of the hepatocyte growth factor receptor allow HBV and HDV replication but have not been shown to be permissive for HCV (496). Other murine models for HBV (73) or woodchuck hepatitis virus (516) may ultimately succeed for HCV, but further work is needed. A single report indicates that a Chinese subspecies of the tupaia or tree shrew, *Tupaia belangeri chinensis*, is susceptible to HCV infection (778). These animals have not achieved widespread use, as they are outbred, caught in the wild, and difficult to maintain and breed in captivity, and HCV infection in them is sporadic even in immunocompromised animals.

Because of their remarkable contribution to the study of HBV pathogenesis (109), transgenic mice with liver-directed expression of HCV proteins have also been created. Thus far, the establishment of a transgenic mouse line that can launch replication-competent HCV RNAs or produce infectious virus particles has not been reported. Mice expressing the HCV capsid protein (also referred to as core or C protein), the structural region or even the entire HCV polyprotein have been derived. Interestingly, at least one line expressing the HCV C protein develops lipid droplets in hepatocytes (i.e., steatosis) and progresses to hepatocellular carcinoma (464,465). This could be related to HCV-associated disease in humans and C-protein-induced lipid droplet formation (25) and transformation (98,300,554,714) in cell cultures. However, studies with other transgenic mice have not confirmed these C-protein-associated effects (317,503,733). Transgenic mice expressing the HCV structural region have been reported to develop spontaneous focal infiltration of lymphocytes and hepatocyte necrosis, and they were more sensitive to liver cell damage induced by injection of anti-Fas antibody (267). In other cases, no pathology was noted (317,336). The Cre/loxP system can be used for activation of HCV transgenes in hepatocytes; it provides an elegant approach for studying the effects of HCV proteins on hepatocyte function and the role of HCV-specific immune responses in pathogenesis (732,733).

Most cell culture studies of HCV have utilized transient transfection protocols or infection with recombinant viruses designed to express HCV proteins. Although such experiments have provided useful information, they may not mimic the situation in HCV-infected hepatocytes. For more than two decades, attempts have been made to propagate the non-A, non-B agent, and later HCV, in various cell cultures. In the postgenomic era of HCV research, when viral RNA can be measured qualitatively and quantitatively, much more has been published on this subject. Continuous hepatoma, B-cell and T-cell lines, primary hepatocytes from humans and chimpanzees, and peripheral blood mononuclear cells (PBMC) have all been reported to support HCV replication (see ref. 29 for review). HCV replication is usually defined by strand-specific reverse transcriptase polymerase chain reaction (RT-PCR) detection of minus-strand RNA. Unfortunately, this technique has been generally unreliable because of false priming during the RT step (225,359,597). Furthermore, accumulation of minus-strand RNA does not necessarily reflect complete replication as opposed to arrest after a single round of minus-strand synthesis. Immunofluorescent detection of HCV antigens, *in situ* hybridization, selection of variant sequences during culture, infection of naive cells or a chimpanzee with cell culture supernate, electron microscopy, inhibition by interferon-α, antisense oligonucleotides, and HCV-specific ribozymes have all been used as indicators of HCV replication in cell culture. Unfortunately, none of the culture systems reported to date have been robust enough to permit classical virologic, biochemical, or genetic dissection of the HCV replication cycle.

One problem that has plagued (and perhaps confused) cell culture infection studies is the limited availability of standardized inocula. As described later, the physical properties of circulating HCV RNA and the specific infectivity of different isolates (RNA molecules per chimpanzee infectious dose, or CID) are highly variable. The best sources of infectious material are acute-phase human or chimpanzee sera that have been titrated for infectivity in chimpanzees. Recently, several laboratories have been making such inocula (often derived from infectious cDNA clones) for each of the major HCV genotypes and subtypes (see ref. 79, and later).

Human hepatitis viruses have been notoriously difficult to grow in cell culture. This presumably results from defects at one or multiple steps of the replication cycle. For positive-strand RNA viruses such as HCV, transfection with infectious RNA can circumvent the entry steps, allowing translation and initiation of RNA replication in permissive cells. Full-length cDNA clones of HCV have been constructed for genotypes 1a, 1b, and 2a and the infectivity of transcribed RNAs validated by intrahepatic inoculation of chimpanzees (36,337,790,791,793). Despite this, attempts to initiate replication by transfection of the same RNAs into cell cultures have not met with success. Such experiments are complicated by a high background of transfected RNA that can persist for months, depending on cell type. Two reports have claimed productive replication after transfection with transcribed RNAs (134,799). These results remain unconfirmed, and it is worth noting that neither study used transcripts containing the correct 3′ NCR. This sequence is the most highly conserved RNA element in the HCV genome, and it was later shown to be required for replication *in vivo* (see later).

A breakthrough for the field was reported in 1999 by Lohmann et al. (397). Based on results for the pestiviruses (43), these investigators engineered bicistronic subgenomic HCV replicons in which the neomycin resis-

tance gene replaced the majority of the HCV structural region and the internal ribosome entry site (IRES) from the encephalomyocarditis virus (EMCV) was used to drive translation of the HCV replicase (beginning with either NS2 or NS3). At low frequency, G418-resistant colonies could be selected that harbored persistently replicating HCV RNAs at a copy number of 500 to 5,000 plus-strand RNAs per cell. More recently, adaptive mutations in the NS5A protein have been identified that allow efficient initiation of HCV RNA replication in as many as 10% of transfected hepatoma cells (51). Thus far, this system has been established for only a single genotype 1b HCV isolate and is restricted to Huh7 cells, a human hepatoma line. Despite this limited host range, we now have a powerful genetic system that should also enhance efforts to identify specific inhibitors of HCV RNA replication. A major gap in our experimental arsenal continues to be the lack of systems that support efficient replication of full-length genome RNAs, particle assembly, and release of infectious virus.

Structure and Physical Properties of the Virion

The size of the infectious virus, based on filtration experiments, is between 30 and 80 nm (62,250,806). HCV particles isolated from pooled human plasma (675), present in hepatocytes from infected chimpanzees, and produced in cell culture (303,535,625,631) have been tentatively visualized by electron microscopy (see Fig. 4F). Initial measurements of the buoyant density of infectious material in sucrose yielded a range of values, with the majority present in a low density pool of less than 1.1 g/mL (60). Subsequent studies have used RT-PCR to detect HCV-specific RNA as an indirect measure of potentially infectious virus present in sera from chronically infected humans or experimentally infected chimpanzees. From these studies, it has become clear that considerable heterogeneity exists between different clinical samples, and that many factors can affect the behavior of particles containing HCV RNA (261,704,745). Such factors include association with immunoglobulins (261,745) or low-density lipoprotein (704,705,745) that may be influenced by genotype (479). In highly infectious acute-phase chimpanzee serum, HCV-specific RNA is usually detected in fractions of low buoyant density (1.03 to 1.1 g/mL) (88,261). In other samples, the presence of HCV antibodies and formation of immune complexes correlate with particles of higher density and lower infectivity (261). Treatment of particles with chloroform, which inactivates infectivity (61,166), or with nonionic detergents, produces RNA-containing particles of higher density (1.17 to 1.25 g/mL) believed to represent HCV nucleocapsids (261,307,446) of about 33 nm (675). The virion protein composition has not been determined, but putative HCV structural proteins include a basic C protein and two membrane glycoproteins, E1 and E2 (see later).

Binding and Entry

For the reasons just stated (lack of standardized infectious stocks, heterogeneous physical properties of virions, and poor infection in cell culture), these steps in the HCV replication cycle have been difficult to study. Even in the infection systems reported, there has been no systematic determination of basic parameters such as optimal adsorption conditions, or requirement for internalization or acidification. Interestingly, it has been shown that bovine and human lactoferrin can block infectivity of HCV in cell culture (288), presumably by binding to virus particles via the E2 glycoprotein (798).

Candidate receptors include the tetraspanin CD81 and the low-density lipoprotein receptor (LDLR). CD81 binds the ectodomain of the E2 glycoprotein and, to a lesser extent, HCV RNA-containing material from infectious plasma (523). The determinants in CD81 responsible for E2 binding reside in the large extracellular loop (LEL), a domain that exhibits species-specific heterogeneity. Specific amino acids in the LEL, important for binding to human and chimpanzee CD81 but not to that of African green monkey, have been mapped (256). However, this initial match between E2–CD81 binding and HCV species tropism is not absolute, as HCV E2 binds even more avidly to tamarin CD81 even though these animals are probably not permissive hosts (10,431). CD81 is expressed on most cell types, including B and T cells, making it unlikely that it is the sole determinant of HCV hepatotropism. Interaction between HCV E2 and CD81-containing signalling complexes on B cells has been postulated to play a role in modulating B-cell activation. This is of interest given the association of HCV with extrahepatic B-cell disorders such as mixed type II cryoglobulinemia and possibly non-Hodgkins B-cell lymphoma (for review, see ref. 570). LDLR has been attractive as a candidate HCV receptor since the discovery of the association between infectious HCV and low-density lipoprotein (LDL) or very low density lipoprotein (VLDL) (3,456,704,705). Coating of virions with serum lipoproteins during their secretion from hepatocytes or after release into circulation could shield the virus from neutralizing antibodies and provide a mechanism for binding and entry that is independent of the HCV glycoproteins. Recent studies indicate that HCV RNA-containing particles can be endocytosed via the LDLR in cell culture (3). What is not clear from the experiments thus far is whether either of these interactions (with CD81 or LDLR) actually leads to productive infection.

In lieu of an efficient infectivity assay, surrogate approaches have been employed. One report utilized vesicular stomatitis virus pseudotypes incorporating cell surface expressed chimeric forms of the HCV glycoproteins (353). Particles expressing either E1 or E2 demonstrated low levels of infectivity that could be neutralized by polyclonal antibodies produced in chimpanzees that

had been immunized with candidate glycoprotein subunit vaccines. Because the two HCV glycoproteins are believed to assemble as heterodimers and mature in the ER (see later), it is unclear if the infectivity being measured in this assay reflects the function of these proteins in the native HCV particle. More recently, a cell–cell fusion assay has been described that requires cell surface expression of both HCV glycoproteins, exhibits low-pH enhanced fusion, and shows some selectivity for certain hepatoma lines (678). Experiments using this system suggest that expression of human CD81 is not sufficient for fusion and that other cell surface proteins and glycosaminoglycans are important.

Genome Structure

The HCV genome is about 9.6 kb in length, considerably shorter than that of flaviviruses. Based on characterization of the HCV translation strategy, and the absence of sequences corresponding to the presumed methyltransferase and RNA triphosphatase enzymes of flaviviruses, it is expected that the HCV genome does not include a 5′ cap structure. The 5′ NCR is a highly conserved RNA sequence element, about 341 nt in length, that biochemical probing and computer modeling indicate is folded into a complex structure consisting of four major domains and a

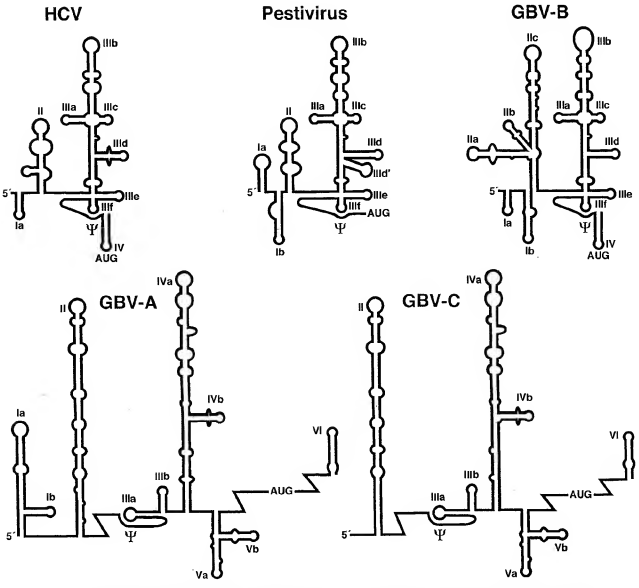


FIG. 9. Predicted secondary structures of pestivirus, hepatitis C virus (HCV), and GB virus internal ribosome entry site (IRES) elements. Model for the secondary and tertiary structures within the 5′ NCRs of HCV, pestiviruses, GB virus A (*GBV-A*), B (*GBV-B*), and C (*GBV-C*). The polyprotein initiation site is indicated by AUG. Major structural domains are labeled *I to VI*. Predicted hairpin loops are indicated by *letters*, and pseudoknots are emphasized with a Ψ symbol. The models are based on computer-assisted folding and mutational analysis. (Courtesy of R. Rijnbrand and S. Lemon. For reviews see ref. 581.)

pseudoknot (Fig. 9) (72,270,645,738). The 5′ NCR probably contains the reverse complement of the information that is read by the replication machinery (i.e., from the 3′ end of viral minus strands) to direct plus-strand RNA initiation. As described later, the 5′ NCR also functions as an IRES to direct cap-independent translation of a single large ORF of about 3,011 codons. The 3′ NCR was initially thought to terminate in polyadenosine (239) or polyuridine (101,106,249,312,498–500,676,685,742,782). However, the previously mentioned studies could not exclude false-priming as a source of these findings. Improved methods for cloning 3′ ends of RNAs later revealed that the HCV 3′ NCR actually consists of a short (about 40-nt) variable domain and a polyuridine/polypyrimidine tract, followed by a highly conserved 98-nt sequence (339,683,684,783). The latter two regions were shown to be essential for recovery of infectious HCV in chimpanzees (340,792). Biochemical probing has not fully resolved the secondary structure of the conserved 98-nt element, although the 3′-most 45-nt sequence has been shown to form a stable stem-loop (52). The HCV 3′ NCR has been shown to interact with several cellular proteins including polypyrimidine tract binding (PTB) protein (197,295,715), which may contribute to the enhanced translational efficiency observed for RNAs containing a 5′ IRES element and the HCV 3′ NCR (296), as well as glyceraldehyde-3-phosphate dehydrogenase (519), and ubiquitous cellular proteins of 87 and 130 kd (289).

Translation and Proteolytic Processing

As mentioned before, the 5′ NCR of HCV has been shown to have IRES activity, directing cap-independent initiation in a number of translation systems (182,270, 287,401,569,578,716,737). The secondary structure of the HCV 5′ NCR (see Fig. 9) provides a framework for understanding the function of the HCV IRES. Although it is unclear whether the approximately 109 5′-proximal nucleotides are necessary for IRES function (568,716), deletion of stem-loop I seems to have a positive effect on translation (270,578,800). The AUG codon used for initiation is precisely defined at a position within stem-loop IV (568,580), the stability of which affects IRES activity (268). The 3′ boundary of the IRES is unclear, with conflicting reports concluding that sequences downstream of the initiation AUG are necessary (270,401,569) or that sequences lacking a complete stem-loop IV are sufficient (716,737). A pseudoknot formed by base-pairing of stem-loop IIIf to a region just upstream of stem-loop IV has been shown to be critical for IRES function (736, and see ref. 581 for further discussion). Interestingly, biochemical reconstitution of internal ribosomal entry for both hepacivirus and pestivirus IRES elements indicated that ribosomal 40S subunits and translational initiation factor eIF3 directly bind to discrete IRES regions, thereby bypassing the need for canonical translation initiation factors eIF4A, eIF4B, and eIF4F (514,515,642). Additional cellular factors that have been shown to bind the HCV IRES, typically enhancing translation, include PTB (8,304), La antigen (9), heterogeneous nuclear ribonucleoprotein L (230), and proteins of 25, 87, and 120 kd (184,797). Furthermore, IRES activity may also involve interaction with one or more cell cycle–specific factors (269).

The organization and processing of the HCV polyprotein is shown in Figure 10. Ten major polypeptides are produced by co- or posttranslational cleavage of the polyprotein and are arranged in the order NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (203–205,379). Cleavages within the structural region (C/E1, E1/E2, and E2/p7) and at the p7/NS2 junction are thought to be mediated by the ER resident cellular signal peptidase, based on the hydrophobicity of regions just upstream of these cleavage sites (258,379,448,449,623), the dependence of cleavage on the presence of membranes (258,282,379,448,449, 602,603) and signal-recognition particles (602,603), and mutagenesis of cleavage sites into suboptimal substrates for signal peptidase (448,449). An additional cleavage event probably removes the E1 signal sequence to produce mature C protein (282,602), although the responsible enzyme has not been definitively identified. Processing at the E2/p7 junction is incomplete, leading to accumulation

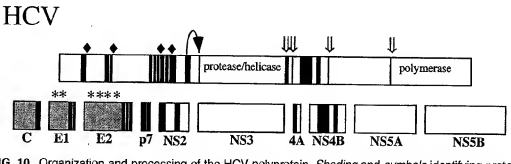


FIG. 10. Organization and processing of the HCV polyprotein. *Shading* and *symbols* identifying proteolytic cleavages are the same as described in Figure 3, except that the *curved arrow* indicates the autocatalytic cleavage at the NS2-3 site catalyzed by the NS2-3 autoprotease.

of E2–p7 (379,448,623). Cleavages in the nonstructural region of the polyprotein are mediated by two virus encoded proteases: the zinc-stimulated NS2-3 autoprotease, which cleaves at the NS2/3 junction (204,259), or the NS3 serine protease (26,152,203,259,407,707), which utilizes NS4A as a cofactor for efficient processing at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites (27,157,380,688).

Features of the Structural Proteins

The C Protein

C is a highly conserved basic protein, appearing as 19- and 21-kd forms (82,205,241,282,390,391,461,553,602, 670,796). The slower-migrating minor form is believed to result from signalase cleavage at only the C/E1 junction, whereas the faster-migrating "mature" form results from cleavage at a second site near residue 173, as discussed before. C protein associates with membranes, particularly the cytoplasmic surface of the ER (99,241,258,326,461, 553,602,670). A minor 16-kd truncated form of C has also been reported by one group (391,392). In terms of HCV particle assembly, binding of C to the HCV 5′ NCR (284,686) has been demonstrated, although nonspecific binding of RNA to C has also been reported (284,602). Multimerization of C, another likely step in the assembly process, has also been observed and requires the N-terminal 115 amino acids of C (423,490). Interaction of C with E1, but not with E2, has been suggested by coprecipitation experiments (393).

Nuclear localization of C, particularly truncated forms lacking C-terminal hydrophobic sequences, has also been reported (99,390,391,553,629,670,796). However, nuclear localization of the intact C protein remains controversial (25,82,461,462,602). Several functions have been proposed for nuclear forms of C protein, including the modulation of cellular gene transcription (555,558,559), repression of transcription from the human immunodeficiency virus 1 (HIV-1) long terminal repeat sequences (649), and the suppression of hepatitis B virus (HBV) transcription and replication in Huh-7 cells (629). This latter effect may be regulated by phosphorylation of C protein at Ser-99 and Ser-116 (628). However, expression of C protein in mice does not appear to inhibit transgene-initiated HBV replication (503). Several additional functions have been proposed for cytoplasmic forms of capsid protein. C protein has been shown to associate with 60S ribosomal subunits (602), which could be involved in the process of virion uncoating. C protein has also been found to associate with lipid droplets and to co-localize with apolipoprotein II (25), which may be related to the observation that the capsid protein has been shown to induce steatosis in transgenic mice (465) or to the reported association of HCV virions with lipids in the bloodstream (704,705). C protein also interacts with the lymphotoxin β receptor (102,422) and tumor necrosis factor receptor 1 (TNFR-1) (815), two members of the TNFR family of cytokine receptors. Interaction with these signalling pathways could be involved in modulating the effectiveness of host antiviral immune responses. In addition, these observations may be related to correlations between C protein expression and cellular sensitivity to apoptosis, although conflicting effects have been reported (102,556,557,585, 815). The C protein has also been reported to transform primary rat embryo fibroblasts (REF) in cooperation with Ras (554) or an immortalized REF cell line called Rat-1 (98). In one study, capsid protein expression in transgenic mice induced hepatocellular carcinoma (464), although such tumors were not reported in other studies of transgenic mice expressing C protein (317,503,733). Thus, although the capsid protein has been the subject of much research, additional work is needed to determine the significance of these observations with respect to specific steps of the viral life cycle and HCV pathogenesis.

Envelope Glycoproteins

HCV E1 and E2, glycoproteins containing type I C-terminal transmembrane anchors, are heavily modified by N-linked glycosylation (205,258,275,283,290,335,336, 360,372,425,426,443,489,547,593,623,648). Based on the extent and type of glycosylation (148,257,360,547, 593,623,648), protection from protease digestion (258, 393), and association with the ER-resident proteins calnexin (114,149), calreticulin (114), immunoglobulin heavy-chain binding protein (BiP) (114), and protein disulfide isomerase (138), E1 and E2 have been shown to be retained within the lumen of the ER. Signals for ER retention have been mapped to the transmembrane domains of both glycoproteins (122,123,150,172). E1 folds slowly and noncovalently interacts with the membrane-proximal domain of E2 to form what are believed to be native heterodimers (123,138,148,149,205,360,426, 443,547). In addition, disulfide-linked aggregates of E1 and E2 have been described (148,205). A valuable tool for studying HCV glycoprotein maturation has been a conformation-sensitive monoclonal antibody, H2, with specificity for noncovalently associated E1E2 heterodimers, which are protease resistant and have been released from the ER chaperone calnexin (138). Kinetic studies with this reagent indicated that stable noncovalent E1E2 complexes form slowly, with a $T_{1/2}$ of about 2 hours, and that formation of native heterodimers is inefficient, accounting for about 5% of the E1E2 complexes (138). The limiting events in complex assembly appear to be formation of intramolecular disulfide bonds in E1 and glycosylation of E1, steps that require coexpression of E2 (147,149,443), as well as productive interaction of the glycoproteins with ER chaperones (114,149,443). Interestingly, calreticulin and BiP preferentially associate with aggregates of misfolded proteins, whereas calnexin is associated with newly synthesized glycoproteins, oxi-

dized monomeric forms, and noncovalent heterodimers (114,149). Overexpression of these three chaperones (using vaccinia virus), either individually or in combination, does not increase the efficiency of productive E1E2 folding (114), suggesting that other unidentified chaperones and folding enzymes may be limiting for proper HCV glycoprotein folding.

As viral glycoproteins, E1 and E2 must have critical interactions with host molecules. It has been noted that E1 residues 264 to 290 bear similarity to suspected or known fusion peptides from flavivirus and paramyxovirus glycoproteins and may perform an analogous function during HCV entry (173). As mentioned, the ectodomain of E2 was shown to bind to the human tetraspanin-family cell surface membrane protein CD81 (523), an interaction that appears to involve a conformationally sensitive region of E2 (171,173) and an extracellular subdomain of CD81 (171,256). This interaction may play a key role in HCV binding and entry, but direct demonstration of this is lacking. In addition, interaction of CD81 with soluble forms of E2 was shown to have antiproliferative effects on lymphocytes (171). The N-terminal portion of E2 contains a hypervariable region (HVR1) (351,493) that is likely to reflect adaptation of the virus to the host immune response and selection of immune escape variants (164,165,315,622,633,750). Indeed, E2 HVR1 has been shown to be a target for neutralizing antibodies (257,314,749,750). Nonetheless, recent work has shown that HVR1 is not essential for replication or chronic infection in chimpanzees, although deletion of this sequence debilitates the virus (175). In addition, expression of E2 has been shown to inhibit the function of the interferon-inducible double-stranded-RNA-activated protein kinase PKR (699), although it is unclear how the proposed interaction involving the E2 ectodomain with cytoplasmic PKR actually occurs.

Features of the Nonstructural Proteins

p7 and NS2

No function is known for the small hydrophobic p7 protein, which is thought to be inefficiently released from the C terminus of E2 by signal peptidase (379,448,449, 623). By analogy with pestivirus p7 (154), HCV p7 and E2–p7 are probably not associated with virions, although an efficient cell culture system is needed to test this.

NS2 (about 23 kd) contains a predicted cysteine protease domain (200), which interacts with the immediately downstream 180 amino acids of NS3 to form the NS2/3 autoprotease that cleaves at the NS2/3 junction (204,259). NS2/3 protease activity is necessary for the *in vivo* infectivity of full-length HCV genomes (340). However, NS2 is dispensable for RNA replication of subgenomic HCV replicons in culture (51,397), and no function is known for NS2 other than in NS2–3 cleavage. NS2/3 activity, which is distinct from the serine protease activity of NS3, is stimulated *in vitro* by addition of microsomal membranes (204,603), various detergents (522), and Zn^{2+} (259). The former two reagents probably allow for proper conformation of the moderately hydrophobic NS2. It is unclear whether Zn^{2+} plays a catalytic or structural role in 2–3 processing. Interestingly, crystallographic (328,400,789) and biochemical (135,658) analyses have identified a site for tetrahedral coordination of Zn^{2+} (HCV polyprotein residues Cys-1123, Cys-1125, Cys-1171, and His-1175) in the region of NS3 required for NS2–3 protease activity, and suggested that this Zn^{2+} ion plays an essential structural role in the NS3 serine protease domain. Consistent with a role for this Zn^{2+} ion in both types of processing, mutations at Cys-1123, Cys-1125, and Cys-1171 inhibit NS2–3 cleavage as well as downstream cleavages catalyzed by the serine protease (259). Mutation of His-1175 has a less dramatic effect (204,259,658), perhaps because this residue indirectly interacts with Zn^{2+} via an H_2O molecule. Although cleavage at the 2/3 site is thought to occur by an autocatalytic mechanism, bimolecular cleavage has been shown to occur, albeit inefficiently, with functional NS2 and/or NS3 protease subunits when coexpressed with substrates containing mutations in either NS2 or NS3 (but not both) that inactivate the NS2–3 protease (204,562). Requirement for a functional NS2 or NS3 region in cis suggests that the overall conformation of NS2/3 may play a role in orienting the NS2/3 cleavage site. In support of this model, mutagenesis of the P5 to P3′ positions at the NS2/3 cleavage site indicated that conformation is a more important determinant for cleavage than primary sequence (263,562).

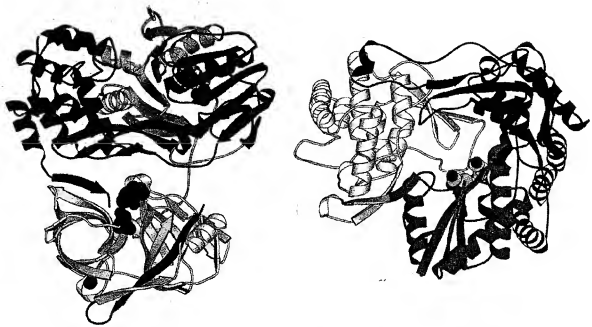
The NS3 Protein

Like the flavivirus NS3 protein, HCV NS3 (about 70 kd) encodes a serine protease domain in the N-terminal one third of the protein, and an NTPase/helicase domain in the C-terminal two thirds. The serine protease is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites (26,152,203,205,259,407,707), and it is required for infectivity of HCV genomes *in vivo* (340). Cleavage at the NS3/4A site occurs in cis, whereas trans cleavage can occur at downstream sites (27,380,689,707). Analysis of cleavage products has revealed that these cleavage sites are highly conserved and conform to the sequence (Asp/Glu)XXXX(Cys/Thr)(Ser/Ala) (152,203, 525). As shown by site-directed mutagenesis and molecular modeling, the P1 residue appears to be an important determinant for cleavage efficiency except at the NS3/4A autocleavage site (28,338,370,525). NS4A is a cofactor for serine protease activity, critical for all serine protease-dependent cleavages except NS5A/5B, although this cleavage can be stimulated by NS4A (27,157,380,690). Cofactor activity requires stable complex formation between

NS3 and NS4A (30,158,381,607), an interaction that also serves to stabilize NS3 and anchor it to cellular membranes (260,690,773).

The structure of the NS3 serine protease domain alone (400), or in complex with an NS4A-derived cofactor (328,789) has been determined by x-ray crystallography. The HCV serine protease structure is similar to other members of the trypsin superfamily, with active site residues and a substrate binding pocket located in a cleft separating two β-barrel domains. Unique to the HCV enzyme, the NS4A cofactor forms an integral part of this structure, interacting with the extreme N-terminal residues of NS3 to form two antiparallel β strands not found in trypsin and altering the geometry of the catalytic site (328,789). These data also reveal an important structural role of the coordinated Zn^{2+} ion for proper folding of the serine protease domain (328,400), a result that is supported by biochemical studies (135,658). Within the predicted substrate-binding pocket, Phe-1180 probably interacts with the conserved Cys residues present in the P1 position of the 4A/4B, 4B/5A, and 5A/5B sites (328,400,525,789). Based on the interaction of NS3 and NS4A, protein engineering studies have created single-chain NS3–NS4A fusion proteins (scNS3–NS4A) with full serine protease activity (144,274,504,692). The structure of full-length NS3 with NS4A cofactor was determined by x-ray crystallography of one scNS3–NS4A (Fig. 11) (795). Folding of the protease and helicase domains of this molecule resembles that of each individual domain (the helicase domain is described later), separated by a single flexible loop (795). Interestingly, the C-terminus of NS3 interacts with the protease active site in a manner consistent with a protease:product (795). Since NS3/4A cleavage occurs autocatalytically, this model provides a unique glimpse at polyprotein cis processing and suggests structural rearrangements that are needed for subsequent trans processing events (795).

HCV NS3 is a member of the Asp-Glu-Cys-His (DECH) subfamily of DEAD-box helicases. The NTPase/helicase activities of NS3 have been demonstrated for recombinant full-length NS3 (188,271,352,463) or the C-terminal two thirds of NS3 (252,301,325,531,669,674). Like the flavivirus enzyme, HCV NS3 ATPase activity is stimulated by single-stranded nucleic acids (227,228,271, 301,305,463,531,669,674). NS3 unwinds RNA and DNA homo- and heteroduplexes in a 3′ to 5′ direction (674), and this requires Mg^{2+} or Mn^{2+} and ATP, suggesting that helicase activity is coupled to ATP hydrolysis (301,463,



A B

FIG. 11. Structures of enzymatic components of the HCV replicase. **A:** The β-barrels of the HCV protease and three subdomains of the helicase are colored from *white to black* along the polypeptide chain. The NS3 protease catalytic triad, the solvent-exposed protease-associated zinc, and a phosphate ion in the helicase NTP binding site are indicated by *dark spheres*, and NS4A is represented by a *black strand*. **B:** The HCV NS5 RNA-dependent RNA (RdRP) polymerase exhibits a globular shape, unique among known polymerase structures. Nevertheless, this viral enzyme retains the typical polymerase subdomain organization consisting of fingers, palm, and thumb subdomains shown in *black*, *gray*, and *white*, respectively. Catalytic residues within the canonical RdRP GDD motif are shown as space-filling *spheres*. In both diagrams, ribbons follow the polypeptide fold and reveal locations of sheets and helices. [Courtesy of N. Yao, C. Lesburg, and P. Weber (see refs. 371 and 795).]

531). However, other NTPs and dNTPs can substitute for ATP in this reaction (301,464), and ATP is not required for RNA binding (188). The crystal structure of the HCV NS3 helicase domain has been determined to a resolution of 2.1 to 2.3 Å in the presence (327) or absence (110,794) of a bound oligonucleotide. These data all reveal three well-defined structural domains surrounding a central axis and separated by distinct clefts (110,327,794). The first two domains, which contain all of the conserved NTPase/helicase motifs, each contain a parallel, six-stranded β sheet surrounded by a number of α helices. However, domain 1 contains a seventh, antiparallel β strand, and domain 2 contains an additional pair of antiparallel β strands that extend into the vicinity of domain 3, which is completely α helical. The catalytic site is located in domain 1 near the cleft that separates it from domain 2. RNA substrate binds in the cleft that separates domains 1 and 2 from domain 3, with the 5′ end near domain 2 (327). Although the precise role of RNA helicases in replication is unknown, mutations in NS3 that disrupt helicase activity ablate HCV infectivity *in vivo* (340).

In addition to its roles in HCV polyprotein processing and RNA replication, several other functions have been proposed for NS3. Cyclic AMP–dependent protein kinase (PKA)-dependent phosphorylation was inhibited by synthetic peptides corresponding to a region of HCV NS3 that exhibits similarity to a PKA inhibitor protein and a PKA autophosphorylation site (56–58). Furthermore, a truncated NS3 protein containing this region was able to interact with the catalytic subunit of PKA and inhibited its forskolin-stimulated nuclear translocation and PKA-catalyzed phosphorylation (57). The serine protease domain of NS3 has been found to have weak transforming activity in NIH-3T3 cells (595) and can suppress actinomycin D–induced apoptosis (180). It is unclear whether these effects may be related to an observed subcellular co-localization of NS3 and the cellular tumor suppressor gene product p53 (294,471).

NS4A and NS4B

The 54-residue NS4A protein (about 8 kd) contains a hydrophobic N-terminal domain followed by a highly charged region. The serine protease cofactor activity of NS4A (see preceding) is contained within a 12-amino-acid region in the central portion of NS4A (30,381,630, 690,708). NS4A also associates with membranes (690, 773), probably through the hydrophobic N-terminal region, and interacts with other replicase components (293,382). NS4B (about 30 kd) is a hydrophobic protein of unknown function.

NS5A

NS5A is a hydrophilic but membrane-associated protein that exists in at least two forms with apparent molecular masses of 56 and 58 kd. Although these forms were originally thought to be the product of alternative proteolysis (260), it is now clear that they result from differential phosphorylation (306,691). NS5A phosphorylation occurs mostly on serine residues and, to a lesser extent, on threonine (306,564). The "basal" phosphorylation site (or sites) of HCV-J NS5A, which is thought to occur for both p56 and p58, has been mapped by serial deletion to the region downstream of polyprotein residue 2350 (690). p58 is "hyperphosphorylated" at additional sites that remain unmodified in the p56 form. Deletion analyses suggest that these hyperphosphorylation sites reside in a conserved, central region of the polyprotein that extends from residue 2200 to residue 2250 (690). Site-directed mutagenesis of the nine conserved serines in this region tentatively identified these sites of p58 phosphorylation as Ser-2197, Ser-2201, and Ser-2204 (690). For an HCV genotype 1a isolate, a major site of *in vitro* and *in vivo* phosphorylation has been mapped to NS5A residue Ser-2321. However, this serine residue is not conserved among the different HCV genotypes (563). For the HCV-J isolate, NS4A associates with NS5A (21) and enhances p58 production and, by inference, hyperphosphorylation at the upstream sites (306,691). However, this effect of NS4A seems to be isolate specific (262) and more recent studies suggest that multiple determinants, including all of the upstream NS proteins, influence NS5A phosphorylation when the protein is expressed in the context of an HCV NS polyprotein (334,389,482).

The kinase responsible for NS5A phosphorylation is thought to be of cellular origin, since (a) NS5A contains no recognizable kinase motifs, (b) phosphorylation of NS5A expressed transiently in cultured cells occurs in the absence of other viral proteins (564,691), and (c) phosphorylation of NS5A expressed in *E. coli* depends on the addition of eukaryotic cell extracts (285,563). Although the identity of the NS5A kinase is not known, biochemical properties of an NS5A-associated kinase activity with similarities to the kinase responsible for NS5A phosphorylation *in vivo* have already been characterized. This NS5A-associated kinase is active *in vitro* over a broad pH range, has an apparent preference for $MnCl_2$ over $MgCl_2$, and is inhibited strongly by $CaCl_2$ at concentrations over 0.5 mM (564). Furthermore, specific inhibitors of PKA and protein kinase C have little or no effect on NS5A phosphorylation *in vitro* or *in vivo*. However, both types of NS5A phosphorylation are inhibited by olomoucine, an inhibitor of certain proline-directed kinases. The resistance of NS5A phosphorylation to treatment with a specific PKA inhibitor casts some doubt on the suggestion that PKA is the major effector of NS5A phosphorylation (285). Although the preference of the NS5A-associated kinase for Mn^{2+} over Mg^{2+} is somewhat unusual among serine/threonine kinases, as is its inhibition by Ca^{2+}, the NS5A and NS5 proteins of bovine viral diarrhea virus (BVDV) and YF, respectively, associate with a kinase that

has similar properties (561). More work is needed to determine the role of NS5A and NS5A phosphorylation in viral replication.

Analysis of full-length genome sequences for three HCV genotype 1b isolates obtained from different Japanese patients before and after interferon therapy found mutations clustered primarily in E2 HVR1 and the C-terminal half of NS5A (155). Further analysis suggested that the amino acid sequence from polyprotein position 2209 to 2248 in NS5A correlated with the effectiveness of interferon treatment in these and other Japanese patients infected with genotype 1b. Consequently, this stretch of amino acids was designated the interferon sensitivity–determining region (ISDR) (155,156). Although confirmed by most other groups working with Japanese patients infected with genotype 1b, 2a, or 2b HCV strains, this corrclation was substantially weaker or lacking in patients infected with genotype 1a strains or European patients infected with strains of genotype 1b, 2b, or 3a (see ref. 508 for review).

In any case, following up on this possible link between NS5A and response to interferon, NS5A was found to interact with and inhibit the interferon-stimulated, double-stranded-RNA-dependent kinase PKR (187). PKR is a major effector of the host antiviral defense pathway that represses translation by phosphorylating the α subunit of the translation initiation factor eIF2. Evidence suggests that HCV NS5A interacts with PKR and inhibits dimerization that is required for PKR activation (185). The region of NS5A implicated in this interaction with PKR includes the ISDR as well as downstream sequences. Several studies have shown that cells expressing NS5A can partially resist the antiviral effects of interferon (186,505,528,647). Somewhat surprisingly, such effects can be observed in the absence of the ISDR (528) and occur independently of the PKR-eIF2 pathway in the context of the complete HCV polyprotein (178). Interestingly, mutations that allow more efficient initiation of HCV subgenomic replicon replication in cell culture cluster in NS5A in the region just upstream of the ISDR and include a 47-amino-acid deletion that encompasses the ISDR (51). These data suggest that NS5A plays an active role in RNA replication and that the ISDR is not essential in cell culture. In addition, deletion of the ISDR does not appear to increase the sensitivity of HCV RNA replication to interferon α in this system (51), consistent with previous observations (528).

N-terminally truncated forms of NS5A fused to the DNA-binding domain of the *Saccharomyces cerevisiae* Gal4 protein activate transcription of reporter genes under the control of promoters containing Gal4 binding sites (119,313,687). This trans-activating ability has been linked to ISDR sequences that may be associated with increased interferon sensitivity (181). The physiologic relevance of these findings is questionable, because the full-length NS5A protein lacks this trans-activating ability and has a cytoplasmic localization. However, NS5A does contain a sequence in its C-terminal region that has the potential to function as a nuclear localization signal (286), raising the possibility that proteolytic removal of the N-terminal region of NS5A (417,463) could result in its transport to the nucleus and activation of the transcription of certain cellular genes. Indeed, recent work has provided evidence for caspase-mediated cleavages in NS5A that liberate a nuclear form whose ability to function as a transcriptional activator is regulated by PKA (606).

In addition to these features, NS5A interacts with several cellular proteins. These include growth-factor-receptor-bound protein-2 adaptor protein, an interaction that can perturb mitogenic signalling (682); a SNARE-like protein, hVAP-33, that is ER and Golgi associated and may participate in intracellular membrane trafficking (717); a novel cellular transcription factor SRCAP (190); and human karyopherin beta3, a protein that may participate in nuclear trafficking of RNAs and proteins (118). The role of these interactions in HCV replication or in virus-induced modulation of host cell function remains to be established.

NS5B

NS5B (about 68 kd) is a hydrophilic protein containing the GDD motif common to RdRPs. As expected, mutation of the polymerase active sites destroy the *in vivo* infectivity of HCV genomes (340) and replication of HCV subgenomes in culture (51,397). RdRP activity has been demonstrated *in vitro* for recombinant NS5B (7,44,396,784,805), although these reactions do not show specificity for HCV templates. DNA or RNA oligonucleotide primers can be extended by the NS5B RdRP on homopolymeric templates or cellular RNAs (6,7, 44,396,784). RNAs terminating with the HCV 3′ NCR, which contains a stable 3′ hairpin (see preceding), can also be utilized as self-priming templates for RNA extension by NS5B (44,396). *De novo*, primer-independent initiation of RNA synthesis has recently been demonstrated in RdRP assays, usually by increasing concentrations of the initiating nucleotide (402,494,814). The more reliable of these studies (402,814) included templates that were chemically blocked at the 3′ end and therefore unable to function as primers. Biochemical characterization of RdRP activity reveals a requirement for the divalent cations Mn^{2+} or Mg^{2+}, but not Zn^{2+} (6,44,167,396,402, 784,805), near neutral pH (7,44,396,784), and low (i.e., 100-mM) concentrations of salt (399,784). The rate of elongation on a genome-length HCV RNA template was estimated to be 150 to 200 nucleotides per minute at 22°C (399,494), and it was independent of NS5B concentration (399), indicating that NS5B is highly processive. Terminal nucleotidyl transferase activity was reported in one preparation of NS5B purified from insect cells (44).

However, several other purified NS5B preparations were apparently free of this activity (7,784,805), suggesting that it is more likely to be due to a copurifying cellular enzyme than an inherent property of NS5B (396).

The hydrophobic C-terminal 21 amino acids can be deleted from NS5B to produce soluble, fully active RdRP (167,396,402,784). This finding greatly enabled several high-resolution structural determinations of NS5B by x-ray crystallography (4,66,371). NS5B bears structural similarity to other polymerases, adopting a "right hand" conformation with a palm subdomain containing active site residues, and discernible fingers and thumb subdomains (see Fig. 11). However, extensive interactions between the fingers and thumb subdomains serve to fully encircle the NS5B active site. Despite the absence of substrate in these experiments, this resembles the closed conformation described for other polymerases (66), suggesting that NS5B may not undergo the typical conformational rotation upon template–primer binding (298,374). Residues thought to be involved in metal ion coordination, substrate binding, and nucleotide discrimination are spatially organized as for HIV-1 reverse transcriptase (4,66,371). Another notable feature of NS5B is a unique β-loop projecting from the thumb subdomain that probably interacts with the template–primer and could affect NS5B fidelity and/or processivity (4,66, 371). The thumb subdomain also contains structural similarity to "armadillo" repeats, which could be involved in mediating protein–protein interactions within the replicase complex (66).

The C-terminal hydrophobic domain of NS5B mediates association with perinuclear membranes, the presumed site of HCV RNA replication (784), and includes determinants for interaction with NS5A (784). Although NS5B binds RNAs nonspecifically, it does show some preference for RNAs containing the HCV 3′ NCR (108,495). NS5B has also been shown to directly interact with NS3 and NS4A (293), which in turn have been shown to form complexes with NS4B and NS5A (21,382). It is likely, yet unproven, that these interactions are important for assembly of a functional HCV RNA replication complex.

RNA Replication

As in other *Flaviviridae*, RNA replication in HCV is likely to occur in association with perinuclear membranes and involve the combined actions of the viral polymerase, helicase, other viral nonstructural proteins, and presumably some host factors (260,293,334,382,482). Because of the lack of an efficient cell culture system, the events of HCV RNA replication are only poorly understood. Detection of viral plus- and minus-strand RNAs in infectious culture systems or clinical samples have been limited to RT-PCR amplification. The most reliable of these methods are designed to eliminate false priming and ensure strand specificity during reverse transcription (225,359). Characterization of HCV subgenomes efficiently replicating in culture by RT-PCR or Northern blot reveals that subgenomes accumulate to 50 to 5,000 copies per cell in these systems (51,397), and that minus strands are 5- to 10-fold less abundant than plus strands (397), which is in agreement with the plus-to-minus-strand ratio observed in infected hepatocytes (359). It has not yet been determined whether viral minus strands can be isolated as RIs or in duplex RFs.

Assembly and Release of Virus Particles

In the absence of an efficient system for culturing HCV, much of what we know about HCV virion assembly comes from heterologous expression studies. Although packaging signals of the HCV genome are not yet characterized, C protein has been shown to interact with stem-loop IIId of the 5′ NCR (634,686). Furthermore, homotypic interactions of C protein (423,490) could be relevant to the formation of viral nucleocapsids. Based on the ER retention of viral glycoproteins E1 and E2, it seems likely that nucleocapsid precursors bud into the ER to acquire a lipid envelope and native heterodimers of the viral glycoproteins, then pass through the host secretory pathway and are released at the cell surface. Formation of virus-like particles (VLPs) by expression of the HCV structural proteins has met with limited success. Presumably nonenveloped (about 30 nm) and enveloped (about 45 nm) VLPs containing C protein were observed in HeLa G cells expressing the full-length HCV coding region via the vaccinia virus-T7 system (447). Enveloped VLPs containing HCV RNA were also observed in insect cells expressing the HCV 5′ NCR and structural region via a recombinant baculovirus (33). These VLPs were observed in intracellular vesicles late in infection (at 72 to 96 hr) but were not secreted and could be released only after mild detergent treatment and sonication. One study indicated that extracellular HCV particles partially purified from human plasma do contain complex N-linked glycans, suggestive of transit through the secretory pathway, although these carbohydrate moieties were not shown to be specifically associated with E1 or E2 (604).

Association of Hepatitis C Virus with HCC

A significant fraction of chronically infected patients slowly progress from chronic active hepatitis to cirrhosis and then to hepato-cellular carcinoma (HCC) (see refs. 54 and 635 and references therein). The mean onset for development of primary HCC has been estimated to be 20 to 30 years (332,594). Studies have identified both positive- and negative-strand HCV RNA in tumorous tissue from some but not all patients. The slow onset and the apparent association with preexisting cirrhosis suggest

that HCV may not directly cause HCC but rather predisposes the organ to carcinogenic events. However, these observations do not exclude the possibility that expression of particular HCV gene products in chronically infected cells might contribute to carcinogenesis. In this regard, expression of C (98,300,554,714), NS3 (595), NS4B (501), and NS5A (186,191) in certain cell cultures can lead to anchorage-independent growth, enhanced formation of colonies in soft agar, and induction of tumor formation in immunodeficient mice. As mentioned earlier, some transgenic mouse lines expressing the C protein develop hepatocellular carcinoma similar in appearance to the human cancer (464). However, the relevance of these observations to the association between HCV and HCC in humans remains to be established.

THE PESTIVIRUSES

Background and Classification

Pestiviruses are animal pathogens of major economic importance, particularly in the livestock industry. They include the type member, bovine viral diarrhea virus, as well as classical swine fever virus (CSFV), formerly known as hog cholera virus, and border disease virus (BDV) of sheep (536). Based on comparative studies, two distinct species of BVDV have recently been identified, BVDV-1 and BVDV-2 (39,42,513,576). Newly described pestiviruses further indicate that additional diversity exists within this genus (39,724). Within the *Flaviviridae*, pestiviruses show greater similarity in genome structure and translation to the hepaciviruses than do the flaviviruses. For this reason, pestivirus research has received increased attention in the search for surrogate model systems to understand the less tractable hepaciviruses.

Pestiviruses are responsible for a spectrum of diseases within their natural hosts (reviewed in ref. 702). CSFV, typically transmitted oronasally, leads to acute or chronic hemorrhagic syndromes with significant mortality. Ruminant pestiviruses, on the other hand, usually cause inapparent or mild symptoms in adult animals. A notable exception is BVDV-2, which is associated with a severe acute hemorrhagic condition in calves (130,513,560, 576). In addition, congenital transmission of pestiviruses can cause fetal death and acute syndromes of the newborn, or it can lead to persistent infection of the offspring in a carrier state. Persistently infected ruminants can be susceptible to a rare but fatal mucosal disease (MD) later in life. Interestingly, development of MD correlates with viral genome alterations that affect the outcome of viral infection on host cells in culture. Recent efforts in pestivirus research have revealed interesting and novel features of these virus–host interactions. Although live attenuated strains and inactivated virus preparations are available for vaccination against CSFV and BVDV (450), there is a need for improved pestivirus vaccines. Insights into pestivirus biology are also being applied to the design of such tools (18,59,77,131,243,588,725,766).

Experimental Systems

In infected animal hosts, viral antigens and infectious virus can be detected in a variety of tissue types including epithelial cells at the site of entry, endothelial cells, lymphoreticular cells, and macrophages. In persistently infected animals, BVDV can be detected in most tissues including PBMC, the gastrointestinal tract, and neurons. Primary and continuous cell lines from natural host species are usually permissive for pestivirus replication in cell culture, although considerable differences in replication efficiencies have been noted (273,588). Highly permissive cell lines have been described for propagation of CSFV (458,588). Infection of permissive tissue culture cells is usually noncytopathic; however, variants of the ruminant pestiviruses capable of causing cytopathic effects can be isolated from animals with mucosal disease. Based on this cell culture phenotype, pestivirus isolates are referred to as either noncytopathogenic (ncp) or cytopathogenic (cp) biotypes.

Complete genome sequences have been determined for a number of pestiviruses (40,71,126,137,139,432,437, 459,460,574,575,586). This has led to the construction of reverse genetic systems (347,430,437,442,459,586,727) that are being used to probe mechanisms of pestivirus replication and the molecular basis of cytopathogenesis and virulence.

Structure and Physical Properties of the Virion

Pestiviruses have been difficult to purify because of modest growth in cell culture, inefficient release from infected cells, and association with cellular debris (362). Identification of efficient culture systems has facilitated visualization of virus particles by electron microscopy (450,746) (see Fig. 4D,E) and the characterization of the structural components of the virion (703). The spherical particles are 40 to 60 nm in diameter and enveloped, and they contain an electron-dense inner core with a diameter of about 30 nm (273). Pestivirus virions band at a buoyant density of 1.134 g/mL in sucrose and are inactivated by heat, organic solvents, and detergents (588). Unlike flaviviruses, which are rapidly inactivated by low pH, pestiviruses can survive over a relatively broad pH range (376). The chemical composition of highly purified preparations of pestivirus particles has not been determined, but in addition to the genome RNA and lipid bilayer, four structural proteins are present. These proteins include capsid protein, C, and three envelope glycoproteins E^{ns} (for ribonuclease, soluble), E1, and E2 (591,703).

Binding and Entry

Based on examples from other viruses, the binding and entry of pestiviruses is likely to be a multistep process involving initial attachment of virions, interaction with specific receptor(s), internalization, and membrane fusion. Specific cell surface receptors for pestiviruses have not been fully characterized. One candidate receptor is a pair of proteins, with apparent relative masses of 60 kd and 93 kd, which are recognized by three monoclonal antibodies capable of blocking infection of several BVDV-1 strains (613). Another potential receptor is a 50-kd cell surface protein identified by using an antiidiotypic antiserum (directed against E2-specific antibodies) that can block binding of BVDV-1 to bovine cells (445,780,781). It has been shown that recombinant E2 and E^{rns} can bind independently to cell surfaces (277). E2 adsorption competitively inhibits infection with homotypic and heterotypic pestiviruses, whereas inhibition by E^{rns} demonstrates virus specificity and requires large amounts of E^{rns} (277). This latter point may be explained by a high receptor density, which correlates with the observed binding of E^{rns} to cell surface glycosaminoglycans (291). Additional information regarding the entry process may come from further characterization of a mutant bovine kidney cell line that is resistant to infection by several pestiviruses but is capable of supporting their replication following RNA transfection or chemically induced virus–cell fusion (174). This cell line was also found to be deficient in expression of the LDLR, a factor putatively involved in hepacivirus binding and entry, and antibodies against this receptor inhibited infection of bovine cells with BVDV-1 (3).

Genome Structure

The pestivirus genome consists of a single-stranded RNA of about 12.3 kb in length (71,126,137,139,432, 460). As discussed later, larger and smaller genome RNAs containing duplications, deletions, and other rearrangements have been found for most cytopathic pestiviruses. The long ORF of about 4,000 codons is flanked by a 5′ NCR of 372 to 385 nucleotides, and a 3′ NCR of 229 to 273 nucleotides (71,126). The 5′ terminus does not appear to contain a cap structure (71,140). Construction of chimeric BVDV genomes containing 5′ NCRs of HCV or EMCV revealed that two 5′-terminal stem-loop structures in the BVDV genome (domains Ia and Ib in Fig. 9) are important for efficient RNA replication, and that the minimal 5′ cis-acting replication element consists of only the terminal tetranucleotide, 5′-GUAU (179). Pestivirus genome RNAs do not contain 3′ poly(A) (126,432,458) but appear to terminate with a short poly(C) tract. The 3′ NCR consists of a variable region near the end of the ORF, followed by a conserved region (140,804). Structural probing revealed that the conserved region forms two hairpins separated by a single-stranded region, and mutational analysis indicated that the terminal hairpin and single-stranded region contain important primary and secondary structural elements that probably function in cis to direct minus-strand initiation (804).

Translation and Proteolytic Processing

Cap-independent translational initiation of the pestivirus genome is mediated by a 5′ IRES element that bears structural and functional similarity to the HCV IRES (see Fig. 9) (72,140,529,579). Structure–function studies have demonstrated that domain I is dispensable for IRES activity, that domains II and III contain structures critical for function, that the 3′ end of the IRES may extend into the ORF, and that a pseudoknot formed from base-pairing of loop IIIf to a region just upstream of the translational start site is necessary for IRES activity (111,529,579). As for hepaciviruses, the pestivirus IRES has been shown to bind ribosomal 40S subunits independent from translation initiation factors eIF4A, eIF4B, and eIF4F (514,515,642). Pestivirus proteins are translated from genomic RNA as a single large polyprotein, which is processed into individual viral proteins (458,543,587). The current model of pestivirus polyprotein processing comes mainly from analysis of virus-infected cells (5, 125,127,591) and expression of pestivirus polyproteins using the vaccinia or baculovirus systems (348,518,590, 591,693,771,779). Cleavage products have been localized in the polyprotein using region-specific antisera (125, 656) and proteolytic processing sites within the polyprotein have been determined by protein sequencing (154, 348,591,655,693,779). The order of the cleavage products in the BVDV-NADL polyprotein is NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Fig. 12) (125,127,439).

Unlike other *Flaviviridae*, the first pestivirus protein encoded in the long ORF is a nonstructural protein, N^{pro}. This autoprotease is responsible for cleavage at the N^{pro}/C site (655,703,771). Processing in the pestivirus structural region appears to be mediated by at least two additional proteases. Although some of the cleavages are slightly delayed, host signal peptidase is believed to cleave at the C/E^{rns} and E1/E2 sites and the site generating the C terminus of E2 (591). The E^{rns}-E1 polyprotein (gp62) is converted slowly to the mature products by an unknown mechanism (591). E1 and E2 are believed to be anchored in the lipid bilayer via C-terminal membrane segments, and some E^{rns} remains associated with the virion via noncovalent interactions that have not been defined (591). A small hydrophobic protein, p7, is produced by signal peptidase, although incomplete processing at the E2/p7 site is observed leading to accumulation of uncleaved E2/p7 (154,242). The pestivirus NS3 protein contains the viral serine protease domain and is responsible for processing

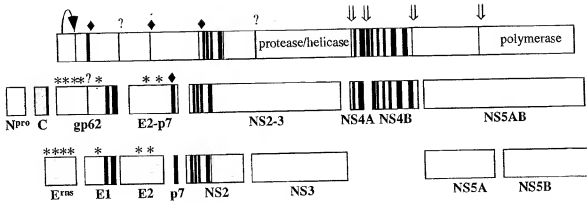


FIG. 12. Processing of the pestivirus polyprotein. *Shading* and *symbols* identifying proteolytic cleavages for the cpBVDV NADL strain are the same as those described in Figure 3, except that the proposed autocatalytic cleavage releasing the N-terminal nonstructural protein N^{pro} from the pestivirus polyprotein (see refs. 655 and 771) is indicated by a *curved arrow.*

at four downstream nonstructural cleavage sites to generate NS4A, NS4B, NS5A, and NS5B (see Fig. 12) (693,771,779). Differences in NS2–3 processing are observed between pestivirus isolates (see ref. 42 and citations therein). Isolates of ncpBVDV do not process NS2-3, whereas those of BDV are able to cleave NS2–3 inefficiently, producing NS3. For CSFV and cpBVDV biotypes, NS2–3 cleavage is efficient but incomplete so that both NS2–3 and NS3 are observed. In the case of the cpBVDV biotypes, the cleavage generating the NS3 N-terminus is produced via several different mechanisms involving RNA recombinational events (detailed later). Enzymes responsible for cleavage at the NS2/3 site in the CSFV and BDV polyproteins have not been elucidated.

Features of Pestivirus Proteins

The N^{pro} Autoprotease

As mentioned earlier, the first protein in the pestivirus polyprotein, the N^{pro} autoprotease (655,771), is a nonstructural protein (703). This enzyme cleaves at its C-terminal sequence Cys↓Ser, which is conserved among pestiviruses (655). Site-directed mutagenesis has identified residues Glu-22, His-49, and Cys-69 as being important for catalysis, and it has been suggested that N^{pro} may be an unusual subtilisin-like cysteine protease (589). Autoproteolysis is the only known function of N^{pro}, and cellular ubiquitin, which directs appropriate C-terminal cleavage by ubiquitin C-terminal hydrolase, can functionally substitute for N^{pro} in replication-competent genomes (43,694,710). Furthermore, N^{pro} is dispensable for autonomous RNA replication in engineered and spontaneously derived subgenomes (43,467,694,710).

Pestivirus Structural Proteins

N^{pro} is followed by the virion nucleocapsid protein C, a conserved, highly basic, 14-kd polypeptide consisting of 21% Lys with a net charge of +12. The C terminus of the virion C protein has not been defined, and it is unknown if it retains the hydrophobic segment postulated to serve as the signal sequence initiating translocation of E^{rns} into the ER lumen.

The E^{rns} glycoprotein (gp44/48, formerly known as E0), is heavily glycosylated at seven to nine potential N-linked glycosylation sites, and it forms disulfide-linked homodimers (703). This protein does not contain a potential membrane-spanning domain and is found noncovalently associated with released virus particles or secreted into the culture medium (591,746,748). Recombinant E^{rns} binds strongly to the surface of cells, probably via interaction with glycosaminoglycans, and it can inhibit infection in a virus-specific manner (291,748). Interestingly, E^{rns} has been shown to possess an unusual ribonuclease activity with specificity for uridine residues (276,620, 767). Glycosylation and dimer formation are not required for this activity (767). Although the function of E^{rns} ribonuclease activity is not yet clear, it appears to be important for some aspect of the virus life cycle. Antibodies that inhibit ribonuclease activity also tend to neutralize virus infectivity (767), and mutations in E^{rns} that destroy ribonuclease activity give rise to viruses that may be more cytopathic in culture but are attenuated *in vivo*

(278,435). In addition, recombinant E^{rns} appears to be toxic to lymphocytes *in vitro* (76), perhaps contributing to the marked leukopenia seen in natural infections (668). Although cytotoxicity is a feature of other solublc ribonucleases (reviewed in ref. 612), it is unclear whether the ribonuclease activity of E^{rns} is related to its toxicity.

Both E1 (gp33) and E2 (gp55) are predicted to be integral membrane proteins and contain two to three, and four to six N-linked glycosylation sites, respectively (see ref. 747). E1 and E2 are associated as disulfide-linked heterodimers that form slowly (591); E2 is also present in homodimers (703,747). As mentioned before, recombinant CSFV E2 can bind to cells and block infection of CSFV and BVDV, suggesting that a common E2 receptor is utilized by these pestiviruses for binding and entry (277). Although the precise roles of the viral glycoproteins in virus assembly and entry remain to be defined, monoclonal antibodies to E^{rns} (746) or E2 (145,506,723, 747,758) can neutralize virus infectivity, and both antigens can elicit protective immunity (279,590,725).

Pestivirus Nonstructural Proteins

Following the virion structural proteins, the first nonstructural protein is p7, and it consists of a central charged region separating hydrophobic termini (154). The role of this small protein is unknown, but it appears to be required for production of infectious virus (242) but not RNA replication (43). Like the hepacivirus protein, pestivirus p7 is inefficiently cleaved from E2, probably via signal peptidase (154,379). Uncleaved E2–p7 is not required for replication in cell culture (242) and both E2–p7 and p7 appear to remain cell associated and do not copurify with virus particles (154).

The NS2 protein (about 54 kd) is present as the N-terminal portion of NS2–3 (about 125 kd) and is found as a mature cleavage product only for some cpBVDV strains (ref. 441 and citations therein). As detailed later, NS2/3 cleavage in cpBVDV can be regulated by several alterations in the NS2 coding region, including genome rearrangements and insertion of cellular sequences. The precise role of NS2 in NS2–3 processing is unclear, and the function of NS2 is largely unknown. Reconstruction of DI-like subgenomes that lack NS2 indicate that it is dispensable for autonomous RNA replication (43,467, 694). However, a correlation between the efficiency of NS2–3 cleavage and the levels of RNA replication has been noted (347,430). NS2 also appears to contain a segment with homology to zinc-finger motifs present in some DNA binding proteins (136).

As for all members of the *Flaviviridae*, the pestivirus NS3 contains an N-terminal serine protease domain (34,91,198,772) followed by motifs characteristic of RNA helicases (199). Uncleaved NS2–3 must be capable of functioning in pestivirus replication, as this protein is not processed in cells infected with ncpBVDV strains. NS3 protease activity (and probably NS2–3 protease activity) requires NS4A as a protein cofactor (779). From cleavage site mapping and comparative analysis, the serine protease was shown to cleave between Leu and small uncharged amino acids L↓(S/A/N) (693,779). Most substitutions of the serine nucleophile eliminate processing at these sites (771,779) and destroy virus infectivity (779). Surprisingly, however, enzymatic activity was retained in mutants containing threonine at this position (695). Additional mutations that disable serine protease activity confirm its essential role in virus viability (207). The NS3 protein of BVDV has been purified and shown to possess RNA helicase (743) and RNA-stimulated NTPase (680) activities. Site-directed mutagenesis of the conserved helicase and NTPase motifs inhibited or destroyed these activities and viral replication coordinately (207,217).

The hydrophobic NS4A and NS4B proteins are similar in size, composition, and hydropathic properties to the NS4A and NS4B proteins of other family members. The only known function of these proteins is the serine protease cofactor activity of NS4A (771,779). As for HCV, cofactor activity involves interaction of a central domain of NS4A with the N-terminal region of NS3 (695).

The remaining two proteins, NS5A (about 58 kd) and NS5B (about 75 kd), are present as mature cleavage products as well as an uncleaved form, NS5AB (about 133 kd) (125,127). Neither protein contains the motifs postulated to be involved in methyltransferase or RNA triphosphatase activities, consistent with the lack of a 5′ RNA cap structure on pestivirus genomes. Little is known about the function of NS5A. This protein was found to be phosphorylated by a cellular serine/threonine kinase with properties similar to enzymes that phosphorylate flavivirus NS5 and hepacivirus NS5A (561). NS5B contains motifs characteristic of RdRPs (124,126). The RNA polymerase activity of recombinant NS5B has been characterized *in vitro* and found to extend template-primed RNAs into double-stranded "copy-back" products (355,398,657,812) or to catalyze *de novo* initiation from short synthetic RNA or DNA templates (308,355). Polymerase activity is particularly responsive to GTP concentration, most likely because of a strong preference for initiation with guanylate (308,398).

RNA Replication

Analysis of pestivirus RNA replication is still at an early stage, although this process appears to be similar to that of flaviviruses. Minus- and plus-strand RNAs have been detected from 4 to 6 hours after infection, followed by the asymmetric accumulation of additional minus- and excess plus-strand RNAs (196). Accumulation of genome-length intracellular pestivirus RNAs, which comigrate with virion RNA, generally follows the time course of infectious virus release, maximal virus titers being achieved about 12 to 24 hours after infection

(196,458,542). Double-stranded RF RNAs and partial duplex RI RNAs, containing about six nascent strands, have been tentatively identified (195,196,542). The role of these RNA isoforms in replication remains to be elucidated.

As described later, there is good evidence that nonhomologous recombination can occur within pestivirus RNAs, and between pestivirus RNAs and host cellular RNAs (reviewed in ref. 441). Although the details of these recombination events are unknown, a likely mechanism is via copy-choice template recruitment during minus-strand synthesis, which is consistent with the coding orientation of cellular inserts. Given the frequency of pestivirus–cellular RNA recombinants compared to other positive-strand viruses, this is an interesting and potentially unusual characteristic of the pestivirus replicase.

Assembly and Release of Virus Particles

Other than the features of the virion structural proteins described before, little information is available on the assembly and release of pestiviruses from infected cells. Pestivirus structural proteins are not found at the plasma membrane (209,748). Electron microscopic examination of virus-infected cells (46,208) suggests that pestiviruses mature in intracellular vesicles and are released by exocytosis. Formation of infectious BVDV-1 particles can be blocked by ER α-glucosidase inhibitors, presumably via misfolding of the viral envelope glycoproteins (816). A substantial fraction of infectious virus remains cell associated and some can be released from infected cells by successive freeze-thaw cycles (361,458). Interestingly, E^{rns} and E2 have been immunolocalized on isolated virus particles by electron microscopy, but E2 was not seen in particles undergoing secretion (or perhaps reattachment) at the cell surface (748). This suggests that E2 may be conformationally inaccessible to antibodies prior to a delayed maturation process.

Pathogenesis of MD and the Generation of Cytopathogenic Pestiviruses via RNA Recombination

Mucosal disease is the most severe outcome of BVDV infection and is usually fatal (reviewed in refs. 23, 74, 75, 441, and 702). This disease can occur when a fetus is infected *in utero* with an ncpBVDV strain. If infection with ncpBVDV occurs between 80 and 100 days of gestation, animals may become tolerized to BVDV antigens and remain persistently infected for life. In the case of a persistently infected animal exhibiting MD, both cp and ncp biotypes of BVDV can be isolated (427). The close serologic relatedness of ncp–cp pairs isolated from an MD-affected animal led to the suggestion that cpBVDV might arise from ncpBVDV by a rare mutational event. Molecular characterization of a number of these ncp–cp pairs has verified this hypothesis and led to the remarkable discovery that some cpBVDV biotypes are generated via RNA recombination (211,434,438,439,698). In every case studied thus far, these rare events led to the production of NS3 (in addition to NS2–3), which is thought to be responsible for cpBVDV cytopathogenicity in cell culture and the pathogenesis of MD in the immunotolerant animal. Although less well characterized, a similar mucosal syndrome correlating with viral cytopathogenicity exists for BDV. Cytopathic CSFV strains have been described but do not seem to correlate with a particular disease state.

Sequence analysis of several independent cpBVDV isolates has revealed some common genome rearrangements resulting from RNA recombination (reviewed in ref. 441), and a few representative genome structures are illustrated in Figure 13. In the case of the Osloss and NADL strains, an in-frame insertion of cellular sequences is found in the NS2 gene (434,438). The Osloss insertion encodes a host ubiquitin monomer, which presumably directs cleavage at the ubiquitin/NS3 junction by the cellular enzyme ubiquitin C-terminal hydrolase. This role for the ubiquitin insertion has been confirmed for another cpBVDV with a similar genome rearrangement (697). The host sequence found in the NADL strain corresponds to a 270-nt portion of a bovine mRNA whose gene product has not been characterized (434). Deletion of this insertion from a cloned BVDV-NADL genome gave rise to a virus that did not produce NS3, was noncytopathic in culture, and exhibited reduced levels of RNA replication (430). Similar insertions of this same host gene have also been found to correlate with NS3 production and cytopathogenicity in two cpBDV isolates (37). Interestingly, a much smaller insertion is found in the NS2 gene of cpBVDV strain CP7, which contains a 27-nt duplication from an upstream region of the NS2 gene in an alternate reading frame (696). This insertion also leads to the production of NS3 (696) and a virus that is cytopathic in culture (437).

The CP1 and Pe515 cpBVDV strains contain large duplications encompassing the NS3 coding region and insertions of either ubiquitin sequences (438,439) or a duplicated copy of the N^{pro} autoprotease (439), respectively. Genome rearrangements with duplicated viral sequences flanking ubiquitin insertion sites have also been noted (545), which probably result from multiple independent recombination events (441). Additional cellular gene insertions have also been found in the context of genomic duplications, and they correlate with NS3 production and cytopathogenicity. These include the SMT3B ubiquitin homolog (544), a ribosomal S27a–ubiquitin gene fusion (41), and light chain 3 of microtubule-associated proteins 1A and 1B (436).

Several cpBVDV-1, cpBVDV-2, and cpCSFV strains consist of a paired DI particle and an ncp helper virus (38,350,440,698,710). An example of a cpBVDV-1 DI is represented by CP9, which contains a precise deletion of

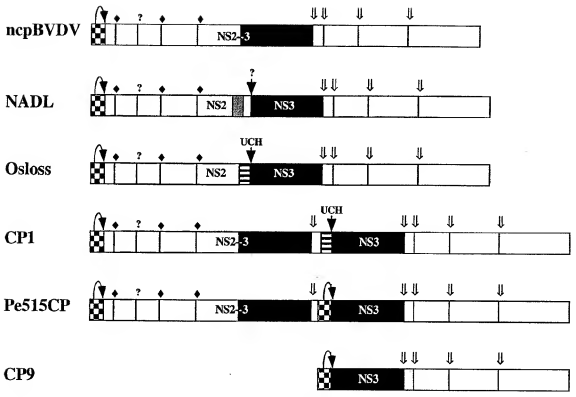


FIG. 13. Genome rearrangements associated with the generation of cytopathogenic bovine viral diarrhea virus (cpBVDV). The *top* diagram indicates the polyprotein of a typical ncpBVDV isolate. *Below*, the polyproteins encoded by five different cpBVDV isolates generated by RNA recombination are shown: NADL (432,433), Osloss (433), CP1 (438), Pe515CP (439), and CP9 (698). For all of the cpBVDV isolates, these polyprotein structures allow the production of both NS2-3 and NS3. In-frame insertions of host sequences are present in NADL (*shaded region*), Osloss (*striped region*), and CP1 (*striped region*). The enzyme responsible for NS3 production in the NADL strain is unknown, but the inserted ubiquitin sequences in Osloss and CP1 provide sites for processing by host ubiquitin C-terminal hydrolase (UCH) (697). For Pe515CP and the CP9 defective-interfering (DI) RNA, the N^{pro} autoprotease (*checkered box*) mediates the cleavage producing the NS3 N-terminus. The nomenclature and organization of the cleavage products and the symbols for the normal processing enzymes are defined in Figures 3 and 10. The location of NS3 (*filled region*) is emphasized.

the C-E^{rns}-E1-E2-p7-NS2 genes resulting in an in-frame fusion of N^{pro} and NS3 (698). Such subgenomes are capable of autonomous replication, express NS3, and induce cytopathic effects within cells (43,467,694). Pestivirus DIs interfere with the replication of helper viruses, which presumably provide packaging functions in trans.

Despite myriad ways that cp pestiviruses can be generated via RNA recombination, a few cpBVDV strains have been described that lack known genome alterations (136,210,512,545). For one such cp strain, BVDV-Oregon, NS2–3 cleavage was shown to be dependent on sequence information contained within NS2 and the first 66 amino acids of NS3 (348). Introduction of this NS2 gene into a heterologous ncpBVDV-1 genome led to NS2–3 processing and cytopathogenicity (347). Surprisingly, a relatively few amino acid changes in NS2 could account for this difference in cleavage efficiency. Nevertheless, due to the lack of an ncp counterpart, it can only be surmised that this strain had been derived by accumulated point mutations in the NS2 region.

Clearly, the unifying feature of cp pestiviruses is the production of NS3. As stated before, except for rearrangements that juxtapose ubiquitin or N^{pro} with NS3, the identity of the protease(s) responsible for NS2–3 cleavage in other cpBVDV strains, as well as in ncpCSFV and ncpBDV, is unknown. Involvement of the NS3 serine protease activity in NS2–3 cleavage has been excluded via mutagenesis or deletion in several cpBVDV polyproteins (348,436,696,779). One possibility is that foreign gene insertions in NS2 might signal a cellular protease to cleave at the NS2/3 junction, perhaps by inducing a conformation within NS2 similar to the NS2 proteins from ncpBDV, ncpCSFV, or BVDV-Oregon, which all cleave NS2–3 despite the absence of genome

alterations. Alternatively, such changes might activate a latent or cryptic protease activity encoded by NS2. This could explain how the BVDV-Oregon NS2 gene is sufficient to direct NS2–3 cleavage, as well as explain NS2–3 cleavage in ncpCSFV and ncpBDV despite the absence of genome rearrangements. Further, in this regard, HCV and GBV-C (see the next section, The GB Viruses) NS2 proteins contain the catalytic residues of NS2–3 autoproteases, although there is scant homology to pestiviruses in this region. Thus if pestivirus NS2 does perform a catalytic role in NS2–3 cleavage, the mechanism is likely to be different from that of these other viruses. Regardless of the mechanistic details, it is interesting to note that pestiviruses differ among themselves and from other family members in NS2–3 cleavage efficiency. Perhaps inefficient cleavage may reflect a recent evolutionary adaptation, producing viruses that are less prone to cause disease but nevertheless successfully spread via congenital routes in domesticated animal herds.

Whereas there is a strong correlation between NS3 production and cytopathogenicity of cpBVDV, ncp strains of BDV and CSFV produce both NS3 and uncleaved NS2–3. This raises the issue of how NS2–3 cleavage leads to a cytopathic effect. One possibility is that NS3 may be directly cytopathic, perhaps involving the serine protease activity. Pestivirus cytopathology proceeds via apoptosis (264,621,809), a cell death pathway involving cellular protease effectors (reviewed in ref. 324). According to this model, the relative abundance of NS3 would determine its effect within a host cell. In this regard, cpBDV and cpCSFV seem to express increased amounts of NS3 (37,42,440). Alternatively, it has been noted that cpBVDV exhibits an increased efficiency of RNA replication compared with paired ncp viruses (347,430). Thus, NS2–3 cleavage might directly result in enhanced RNA replication, and a byproduct of this process could be responsible for cytopathogenicity. In this regard, enhanced levels of RI and RF RNA forms could activate the double-stranded RNA-activated kinase PKR, a known inducer of apoptosis (24,141,652). A larger question is how cytopathic effects on the cellular level lead to MD in infected animals. Increased cell death could directly contribute to tissue injury and induce inflammation. There is also evidence that animals with MD show increased numbers of infected cells (375), perhaps because of increased replication of cpBVDV. In addition, cpBVDV might exhibit differences in tropism that could contribute to disease (375).

THE GB VIRUSES

Discovery, Distribution, and Origin

In the early 1990s, a residual number of hepatitis cases were still not attributable to HAV, HBV, HCV, or the recently described hepatitis E virus (Chapter 89). Efforts aimed at identifying additional agents of hepatitis revealed three novel viruses that have been tentatively assigned to the *Flaviviridae*. Two of these viruses, GBV-A and GBV-B, were cloned via subtractive representational methods from the serum of a tamarin experimentally infected with a hepatitis agent originally derived from a human patient, GB (641). Although some human cases of non-A–E hepatitis showed serologic reactivity to GBV-A and GBV-B, RT-PCR analysis from these patients failed to detect either virus. Rather, a third related virus, GBV-C, was subsequently identified from one such patient (365,640). Cloning of novel viruses associated with non-A, non-B hepatitis by immunoscreening of a cDNA library identified an agent, initially termed hepatitis G virus (HGV), which turned out to be an independent isolate of GBV-C (386).

Based on sequence relatedness and overall genome structure, GBVs have been categorized within the *Flaviviridae*, although they remain unclassified at the genus level. GB viruses are most similar to HCV but phylogenetically distant enough to resist classification as hepaciviruses (see Fig. 1) (78,582). This is especially true for GBV-A and GBV-C, which share a number of unique features that distinguish them from other members of the family.

The inability to detect GBV-A or GBV-B in human samples led to investigations into their origins. Interestingly, GBV-A has been detected in several species of New World monkeys in the absence of experimental infection or overt disease (78,100,364,615). Viral sequences isolated from within a single primate species were highly related, whereas sequences isolated from separate species showed greater divergence, indicating that GBV-As are indigenous monkey viruses that have adapted to their hosts over extended periods of time (78,100,364,615). These results, and observations made during early cross-challenge experiments, provide support for the view that the original GBV-A isolate may have been acquired during passage of the GB agent in tamarins (78,502). The distribution of GBV-B in nature is unknown, as the only source of this virus is the original tamarin-passaged GB serum used to identify GBV-A and GBV-B.

Since its initial discovery, GBV-C has been found to be surprisingly common, with viral RNA detected in about 1% to 4% of healthy human volunteer blood donors (168,421,654,775). Phylogenetic analysis of GBV-C sequences has been complicated by an apparent bias against synonymous substitution in some parts of the genome, leading to differences in inferred relationships depending on the subgenomic regions under comparison (309,521,728). The molecular basis for this bias is unclear; it may involve evolutionary constraints imposed by RNA secondary structures (637) or cryptic ORFs (507,643). Nevertheless, standardization of results has been achieved through the use of an appropriate molecular timepiece, such as the 5′ NCR, the E2 gene, or com-

plete genome sequences. These results indicate that variation among GBV-C isolates occurs in distinct genotypes that reflect their geographical distribution (183, 387,469,643,644). GBV-C RNA has also been detected in the serum of wild chimpanzees (1) and chimpanzees infected with a putative hepatitis agent (48). Chimpanzee-infecting GBV-Cs were found to be more closely related to each other than to human-infecting GBV-Cs, suggesting coevolution of these viruses with their hosts (1,48,100).

Genome Structure and Expression

Complete or nearly complete genome sequences have been determined for a number of GBVs, and functional full-length infectious clones have been assembled for GBV-B (80) and GBV-C (777). Like the other family members, the GBV genomes encode a single long ORF containing structural protein genes [(C)-E1-E2] in the 5′ one third, followed by nonstructural protein genes (NS2-NS3-NS4A-NS4B-NS5A-NS45B) in the remainder (365,468). The 5′ NCR of GBV-B contains 445 nucleotides, which is about 30% larger than the HCV 5′ NCR, but it has significant similarity in primary and presumed secondary structure to the HCV 5′ NCR (see Fig. 11) (268,468). In contrast, GBV-A and GBV-C have 5′ NCRs of 508 to 593 nucleotides that share about 50% identity to each other and can be folded into similar structures (see Fig. 11), but that do not resemble the 5′ NCRs of HCV or GBV-B (639). The long 5′ NCRs of all three GBVs contain multiple initiator AUG codons, and initiation of polyprotein translation is thought to be dependent on IRES activities, which have been demonstrated for all three GBV 5′ NCRs (201,577,639). The GBV-B 3′ NCR is 361 nucleotides in length, containing a short poly(U) stretch 30 nucleotides downstream of the stop codon, followed by 309 nucleotides of unique sequence (80,609). Although this region of the GBV-B genome does not contain sequence homology with HCV, the 3′ 47 nucleotides of GBV-B is predicted to fold into a structure very similar to SL-1 at the 3′ end of the HCV genome (80,609). The 3′ NCRs of GBV-A and GBV-C are highly conserved only within these virus groups, although a short 17-nucleotide stretch is also well conserved among all GBVs (609).

The relationships among GBVs and between HCV are also reflected in the organization of structural proteins. Like HCV, GBV-B encodes a basic capsid protein followed by two envelope glycoproteins. The genomes for GBV-A and GBV-C also contain E1 and E2 glycoproteins, but they lack any obvious capsid-like protein (386,468). *In vitro* translation of RNAs containing the GBV-A or GBV-C 5′ NCR localized the translational start site to a conserved AUG immediately upstream of the E1 coding region (639). However, it has been observed that GBV-C-infected humans generate antibody responses against a small basic peptide that can be translated from an in-frame upstream AUG, suggesting that such a protein is expressed *in vivo* (777). Alternative explanations for the lack of a capsid-like protein include the possibilities that GBV-A and GBV-C might usurp a capsid-like protein of the host cell or a co-infecting virus, or that additional GBV proteins may be involved. In this regard, a region of the GBV-C NS5A gene exhibiting a bias against synonymous substitution has been noted to potentially encode a small basic protein (10 kd, pI 11.5) in an alternate reading frame (507). Furthermore, it remains possible that GBV-A and GBV-C virions might lack a distinct nucleocapsid. However, biophysical characterization of GBV-C particles indicate that GBV-C RNA can be found in low-density (1.07 g/mL) and intermediate-density (about 1.18 g/mL) fractions on a variety of density gradients (428,605,777). Pretreatment with detergents or organic solvents to remove membranes shifts the peak of viral RNA to a higher-density form that may represent nucleocapsids that have recently been visualized by electron microscopy (776).

The nonstructural proteins of GBVs show the greatest similarity to HCV, and the boundaries of cognate NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins have been proposed (365,468). Catalytic residues of the HCV NS2-3 autoprotease are conserved among GBV NS2 proteins (365,468), and this enzymatic activity has been described for GBV-C (45). Similarly, active sites of the HCV NS3 serine protease are retained in NS3 of GBVs. Biochemical characterization of the GBV-B serine protease activity indicates that it shares substrate specificity with the HCV enzyme and requires the virus-specific cofactor NS4A (83,610). The C-terminal region of GBV NS3 proteins retain the similarity to supergroup 2 RNA helicases that is common to the *Flaviviridae* (365,468), and NTP-dependent RNA unwinding activity has been demonstrated for NS3 proteins of GBV-B and GBV-C (229,813). NS5B contains similarity to the supergroup 2 RNA polymerases (365,468,641).

Association with Disease?

GBV-A has not been shown to cause disease in nonhuman primates, whereas GBV-B causes hepatitis in experimentally infected tamarins (80,614). As described before, although both of these viruses were thought to be derived from a source of human hepatitis, it appears that GBV-A had been acquired during primate passage and GBV-B has been isolated only from tamarin-passaged GB material. Attempts to identify a GBV in the original GB clinical sample have failed, most likely because of degradation during storage (614).

Human infection with GBV-C is well documented, although direct association of this virus with human disease has proven to be elusive (reviewed in ref. 638). Epidemiologic evidence suggests that it is primarily blood-

borne, although other modes of transmission appear possible (105,169,240,624,654). These risk factors overlap with those of HBV and HCV, and the frequency of coinfections with GBV-C and other viruses have complicated etiologic determinations (13,386). It appears that a majority of GBV-C infections are subclinical, with only mild symptoms that typically resolve with the appearance of anti-E2 antibody (143,226,368,369,672,673). Viral persistence seems to occur in about 5% to 10% of GBV-C infections (226,421). A few studies have implicated GBV-C in acute and chronic non-A–E hepatitis (168), fulminant hepatitis (679,801,802), or other liver disease (129). However, recent work suggests that GBV-C is a lymphotropic virus (718). Clearly, more work needs to be done to establish the clinical significance of GBV-C and related viruses.

SUMMARY AND QUESTIONS

Although much has been learned about the general life cycle of the *Flaviviridae*, it is quite complex, and large gaps in our knowledge exist for every step. The ongoing development of improved genetic and biochemical tools to study these viruses will certainly enable a more complete picture of their biology. Recent progress has been made in identifying host cell surface molecules that could be involved in binding and entry of viruses, although the details of such interactions, and how these control virus tropism and infection *in vivo*, is largely unknown. The general strategies of genome translation and polyprotein processing have been elucidated. Yet details such as the proteolytic processing mechanism of the flavivirus NS1-2A or pestivirus NS2-3 polypeptides need to be resolved. More effort is needed to understand the functional significance of polyprotein processing events for RNA replication, virus–host interaction, and virion formation. Furthermore, it is not understood how the competing processes of genome translation and genome replication are regulated for this virus family. New insights have emerged regarding the enzymology of some viral nonstructural proteins, as well as the identity of a few host proteins that most likely contribute to genome replication. However, the role of several nonstructural proteins in this process are unknown. More description is needed for how all these components, together with viral RNA, combine to produce a functional replicase. As for all positive-strand RNA viruses, the role of membranes in the process of RNA replication remains a mystery. It is interesting that phosphorylation of polypeptides upstream of the polymerase seems to be a conserved feature within the family. Identification of the relevant kinase(s) should allow the significance of this posttranslational modification to be addressed. Nearly all of what we know about virus–cell interaction for HCV has come from the study of heterologous systems, which have suggested important roles for HCV-specific gene products in interferon sensitivity and the development of HCC. The emergence of improved systems for studying HCV in culture will permit all of this work to be reevaluated, and moreover, it will undoubtedly reveal unexpected and novel aspects of HCV biology. Our understanding of virion formation is still at an early stage, and it is not yet clear how structural proteins combine to form nascent virions, where this process occurs, and whether packaging is temporally regulated within the replication cycle. The lack of obvious capsid proteins for GBV-A and GBV-C raises the questions of whether these virions actually contain nucleocapsids and, if so, what the identity of the capsid proteins is. It is fascinating that pestiviruses encode a ribonuclease within the extracellular domain of an envelope glycoprotein, although the biologic role of this activity remains to be clarified. Also, what is the role of the secreted flavivirus NS1 protein? Why does production of NS3 by cpBVDV correlate with cytopathogenicity and fatal MD? Are GBVs involved in human disease? What mechanisms are involved in the establishment and maintenance of chronic infections by pestiviruses and HCVs? Answers to these and other pressing questions should reveal the unique aspects to the replication of this evolutionarily distinct family of viruses. This will provide information useful for the development of effective immunization and therapeutic strategies to control diseases caused by these diverse and important pathogens. Clearly, the most exciting period of research into the *Flaviviridae* lies ahead.

REFERENCES

1. Adams NJ, Prescott LE, Jarvis LM, et al. Detection in chimpanzees of a novel flavivirus related to GB virus-C/hepatitis G virus. *J Gen Virol* 1998;79:1871–1877.
2. Agnello V. Mixed cryoglobulinemia and other extrahepatic manifestations of hepatitis C virus infection. In: Liang TJ, Hoofnagle JH, eds. *Hepatitis C*. San Diego: Academic Press, 2000:295–314.
3. Agnello V, Abel G, Elfahal M, Knight GB, Zhang Q-X. Hepatitis C virus and other *Flaviviridae* viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* 1999;96:12766–12771.
4. Ago H, Adachi T, Yoshida A, et al. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Structure Fold Des* 1999;7:1417–1426.
5. Akkina RK. Pestivirus bovine viral diarrhea virus polypeptides: Identification of new precursor proteins and alternative cleavage pathways. *Virus Res* 1991;19:67–81.
6. Al RH, Xie Y, Wang Y, Hagedorn CH. Expression of recombinant hepatitis C virus non-structural protein 5B in *Escherichia coli*. *Virus Res* 1998;53:141–149.
7. Al RH, Xie Y, Wang Y, et al. Expression of recombinant hepatitis C virus NS5B. *Nucleic Acids Symp Ser* 1997;36:197–199.
8. Ali N, Siddiqui A. Interaction of polypyrimidine tract-binding protein with the 5′ noncoding region of the hepatitis C virus RNA genome and its functional requirement in internal initiation of translation. *J Virol* 1995;69:6367–6375.
9. Ali N, Siddiqui A. The La antigen binds 5′ noncoding region of the hepatitis C virus RNA in the context of the initiator AUG codon and stimulates internal ribosome entry site-mediated translation. *Proc Natl Acad Sci U S A* 1997;94:2249–2254.
10. Allander T, Forns X, Emerson SU, et al. Hepatitis C virus envelope protein E2 binds CD81 of tamarins. *Virology* 2000;277:358–367.
11. Allison SL, Schalich J, Stiasny K, et al. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J Virol* 1995;69:695–700.

12. Allison SL, Stiasny K, Stadler K, et al. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. *J Virol* 1999;73:5605–5612.
13. Alter HJ. The cloning and clinical implications of HGV and HGBV-C [editorial; comment]. *N Engl J Med* 1996;334:1536–1537.
14. Alter HJ, Purcell RH, Holland PV, Popper H. Transmissible agent in non-A, non-B hepatitis. *Lancet* 1978;1:459–463.
15. Alter HJ, Seeff LB. Recovery, persistence and sequelae in hepatitis C virus infection: A perspective on the long-term outcome. *Semin Liver Dis* 2000;20:17–25.
16. Amberg SM, Nestorowicz A, McCourt DW, Rice CM. NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: *In vitro* and *in vivo* studies. *J Virol* 1994;68:3794–3802.
17. Anderson JF, Andreadis TG, Vossbrinck CR, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. *Science* 1999;286:2331–2333.
18. Andrew ME, Morrissy CJ, Lenghaus C, et al. Protection of pigs against classical swine fever with DNA-delivered gp55. *Vaccine* 2000; 18:1932–1938.
19. Anonymous. World Health Organization—Hepatitis C: Global prevalence. *Wkly Epidemiol Rec* 1997;72:341–344.
20. Arias CF, Preugschat F, Strauss JH. Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. *Virology* 1993;193:888–899.
21. Asabe S-I, Tanji Y, Satoh S, et al. The N-terminal region of hepatitis C virus-encoded NS5A is important for NS4A-dependent phosphorylation. *J Virol* 1997;71:790–796.
22. Asnis D, Conetta R, Waldman G, et al. Outbreak of West Nile-like Viral Encephalitis—New York, 1999. *MMWR Morb Mortal Wkly Rep* 1999;48:845–849.
23. Baker JC. Bovine viral diarrhea virus: A review. *J Am Vet Med Assoc* 1987;190:1449–1458.
24. Balachandran S, Kim CN, Yeh WC, et al. Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling. *EMBO J* 1998;17:6888–6902.
25. Barba G, Harper F, Harada T, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A* 1997;94:1200–1205.
26. Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H. Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J Virol* 1993; 67:3835–3844.
27. Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J Virol* 1994;68:5045–5055.
28. Bartenschlager R, Ahlborn-Laake L, Yasargil K, et al. Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. *J Virol* 1995;69:198–205.
29. Bartenschlager R, Lohmann V. Replication of hepatitis C virus. *J Gen Virology* 2000;81:1631–1648.
30. Bartenschlager R, Lohmann V, Wilkinson T, Koch JO. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. *J Virol* 1995;69:7519–7528.
31. Bartholomeusz AI, Wright PJ. Synthesis of dengue virus RNA *in vitro*: Initiation and the involvement of proteins NS3 and NS5. *Arch Virol* 1993;128:111–121.
32. Bassett SE, Brasky KM, Lanford RE. Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profiles. *J Virol* 1998;72:2589–2599.
33. Baumert TF, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* 1998;72:3827–3836.
34. Bazan JF, Fletterick RJ. Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. *Virology* 1989;171:637–639.
35. Bazan JF, Fletterick RJ. Structural and catalytic models of trypsin-like viral proteases. *Semin Virol* 1990;1:311–322.
36. Beard MR, Abell G, Honda M, et al. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* 1999;30: 316–324.
37. Becher P, Meyers G, Shannon AD, Thiel H-J. Cytopathogenicity of border disease virus is correlated with integration of cellular sequences into the viral genome. *J Virol* 1996;70:2992–2998.
38. Becher P, Orlich M, Konig M, Thiel HJ. Nonhomologous RNA recombination in bovine viral diarrhea virus: Molecular characterization of a variety of subgenomic RNAs isolated during an outbreak of fatal mucosal disease. *J Virol* 1999;73:5646–5653.
39. Becher P, Orlich M, Kosmidou A, et al. Genetic diversity of pestiviruses: Identification of novel groups and implications for classification. *Virology* 1999;262:64–71.
40. Becher P, Orlich M, Thiel H-J. Complete genomic sequence of border disease virus, a pestivirus from sheep. *J Virol* 1998;72:5165–5173.
41. Becher P, Orlich M, Thiel HJ. Ribosomal S27a coding sequences upstream of ubiquitin coding sequences in the genome of a pestivirus. *J Virol* 1998;72:8697–8704.
42. Becher P, Shannon AD, Tautz N, Thiel H-J. Molecular characterization of border disease virus, a pestivirus from sheep. *Virology* 1994;198: 542–551.
43. Behrens S-E, Grassmann CW, Thiel H-J, et al. Characterization of an autonomous subgenomic pestivirus RNA replicon. *J Virol* 1998;72: 2364–2372.
44. Behrens SE, Tomei L, DeFrancesco R. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J* 1996;15:12–22.
45. Belyaev AS, Chong S, Novikov A, et al. Hepatitis G virus encodes protease activities which can effect processing of the virus putative nonstructural proteins. *J Virol* 1998;72:868–872.
46. Bielefeldt Ohmann H, Bloch B. Electron microscopic studies of bovine viral diarrhea virus in tissues of diseased calves and in cell cultures. *Arch Virol* 1982;71:57–74.
47. Bielefeldt-Ohmann H. Analysis of antibody-independent binding of dengue viruses and dengue virus envelope protein to human myelomonocytic cells and B lymphocytes. *Virus Res* 1998;57:63–79.
48. Birkenmeyer LG, Desai SM, Muerhoff AS, et al. Isolation of a GB virus-related genome from a chimpanzee. *J Med Virol* 1998;56:44–51.
49. Blackwell JL, Brinton MA. BHK cell proteins that bind to the 3′ stem-loop structure of the West Nile virus genome RNA. *J Virol* 1995;69: 5650–5658.
50. Blackwell JL, Brinton MA. Translation elongation factor-1 alpha interacts with the 3′ stem-loop region of West Nile virus genomic RNA. *J Virol* 1997;71:6433–6444.
51. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000;290:1972–1974.
52. Blight KJ, Rice CM. Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA. *J Virol* 1997;71:7345–7352.
53. Blitvich BJ, Scanlon D, Shiell BJ, et al. Identification and analysis of truncated and elongated species of the flavivirus NS1 protein. *Virus Res* 1999;60:67–79.
54. Blum HE. Does hepatitis C virus cause hepatocellular carcinoma? *Hepatology* 1994;19:251–255.
55. Boege U, Heinz FX, Wengler G, Kunz C. Amino acid compositions and amino-terminal sequences of the structural proteins of a flavivirus, European tick-borne encephalitis virus. *Virology* 1983;126: 651–657.
56. Borowski P, Heiland M, Oehlmann K, et al. Non-structural protein 3 of hepatitis C virus inhibits phosphorylation mediated by cAMP-dependent protein kinase. *Eur J Biochem* 1996;237:611–618.
57. Borowski P, Oehlmann K, Heiland M, Laufs R. Nonstructural protein 3 of hepatitis C virus blocks the distribution of the free catalytic subunit of cyclic AMP-dependent protein kinase. *J Virol* 1997;71: 2838–2843.
58. Borowski P, zur Wiesch JS, Resch K, et al. Protein kinase C recognizes the protein kinase A-binding motif of nonstructural protein 3 of hepatitis C virus. *J Biol Chem* 1999;274:30722–30728.
59. Bouma A, de Smit AJ, de Kluijver EP, et al. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. *Vet Microbiol* 1999;66:101–114.
60. Bradley D, McCaustland K, Krawczynski K, et al. Hepatitis C virus: Buoyant density of the factor VIII-derived isolate in sucrose. *J Med Virol* 1991;34:206–208.
61. Bradley DW, Maynard JE, Popper H, et al. Posttransfusion non-A, non-B hepatitis: Physicochemical properties of two distinct agents. *J Infect Dis* 1983;148:254–265.
62. Bradley DW, McCaustland KA, Cook EH, et al. Posttransfusion non-A, non-B hepatitis in Chimpanzees: Physicochemical evidence that the tubule-forming agent is a small, enveloped virus. *Gastroenterology* 1985;88:773–779.

63. Brandt WE, Chiewsilp D, Harris DL, Russell PK. Partial purification and characterization of a dengue virus soluble complement-fixing antigen. *J Immunol* 1970;105:1565–1568.
64. Brawner JA, Trousdale MD, Trent DW. Cellular localization of Saint Louis encephalitis virus replication. *Acta Virol* 1979;23:284–294.
65. Bray M, Men R, Lai CJ. Monkeys immunized with interfypic chimeric dengue viruses are protected against wild-type virus challenge. *J Virol* 1996;70:4162–4166.
66. Bressanelli S, Tomei L, Roussel A, et al. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci U S A* 1999;96:13034–13039.
67. Brinton MA. Analysis of extracellular West Nile virus particles produced by cell cultures from genetically resistant and susceptible mice indicates enhanced amplification of defective interfering particles by resistant cultures. *J Virol* 1983;46:860–870.
68. Brinton MA. Replication of flaviviruses. In: Schlesinger S, Schlesinger MJ, eds. *The Togaviridae and Flaviviridae*. New York: Plenum Press, 1986:327–365.
69. Brinton MA, Dispoto JH. Sequence and secondary structure analysis of the 5'-terminal region of flavivirus genome RNA. *Virology* 1988;162:290–299.
70. Brinton MA, Fernandez AV, Amato J. The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* 1986; 153:113–121.
71. Brock KV, Deng R, Riblet SM. Nucleotide sequencing of 5' and 3' termini of bovine viral diarrhea virus by RNA ligation and PCR. *J Virol Methods* 1992;38:39–46.
72. Brown EA, Zhang H, Ping LH, Lemon SM. Secondary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. *Nucleic Acids Res* 1992;20:5041–5045.
73. Brown JJ, Parashar B, Moshage H, et al. A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice. *Hepatology* 2000; 31:173–181.
74. Brownlie J. Pathogenesis of mucosal disease and molecular aspects of bovine virus diarrhoea virus. *Vet Microbiol* 1990;23:371–382.
75. Brownlie J, Clarke MC. Experimental and spontaneous mucosal disease of cattle: A validation of Koch's postulates in the definition of pathogenesis. *Intervirology* 1993;35:51–59.
76. Bruschke CJ, Hulst MM, Moormann RJ, et al. Glycoprotein E^{rns} of pestiviruses induces apoptosis in lymphocytes of several species. *J Virol* 1997;71:6692–6696.
77. Bruschke CJ, van Oirschot JT, van Rijn PA. An experimental multivalent bovine virus diarrhea virus E2 subunit vaccine and two experimental conventionally inactivated vaccines induce partial fetal protection in sheep. *Vaccine* 1999;17:1983–1991.
78. Bukh J, Apgar CL. Five now or recently discovered (GBV-A) virus species are indigenous to New World monkeys and may constitute a separate genus of the *Flaviviridae*. *Virology* 1997;229:429–436.
79. Bukh J, Apgar CL, Engle R, et al. Experimental infection of chimpanzees with hepatitis C virus of genotype 5a: Genetic analysis of the virus and generation of a standardized challenge pool. *J Infect Dis* 1998;178:1193–1197.
80. Bukh J, Apgar CL, Yanagi M. Toward a surrogate model for hepatitis C virus: An infectious molecular clone of the GB virus-B hepatitis agent. *Virology* 1999;262:470–478.
81. Bukh J, Miller RH, Purcell RH. Genetic heterogeneity of hepatitis C virus: Quasispecies and genotypes. *Semin Liver Dis* 1995;15:41–63.
82. Buratti E, Baralle FE, Tisminetzky SG. Localization of the different hepatitis C virus core gene products expressed in COS-1 cells. *Cell Mol Biol (Noisy-le-grand)* 1998;44:505–512.
83. Butkiewicz N, Yao N, Zhong W, et al. Virus-specific cofactor requirement and chimeric hepatitis C virus/GB virus B nonstructural protein 3. *J Virol* 2000;74:4291–4301.
84. Cahour A, Pletnev A, Vazielle FM, et al. Growth-restricted dengue virus mutants containing deletions in the 5' noncoding region of the RNA genome. *Virology* 1995;207:68–76.
85. Calisher CH. Antigenic classification and taxonomy of flaviviruses (family *Flaviviridae*) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis [see comments]. *Acta Virol* 1988;32:469–478.
86. Calisher CH, Karabatsos N, Dalrymple JM, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 1989;70:37–43.
87. Cardiff RD, Russ SB, Brandt WE, Russell PK. Cytological localization of dengue-2 antigens: An immunological study with ultrastructural correlation. *Infect Immun* 1973;7:809–816.
88. Carrick RJ, Schlauder GG, Peterson DA, Mushahwar IK. Examination of the buoyant density of hepatitis C virus by the polymerase chain reaction. *J Virol Methods* 1992;39:279–289.
89. Cauchi MR, Henchal EA, Wright PJ. The sensitivity of cell-associated dengue virus proteins to trypsin and the detection of trypsin-resistant fragments of the nonstructural protein NS1. *Virology* 1991;180: 659–667.
90. Chambers TJ, Grakoui A, Rice CM. Processing of the yellow fever virus nonstructural polyprotein: A catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. *J Virol* 1991;65:6042–6050.
91. Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* 1990;44: 649–688.
92. Chambers TJ, McCourt DW, Rice CM. Yellow fever virus proteins NS2A, NS2B, and NS4B: Identification and partial N-terminal amino acid sequence analysis. *Virology* 1989;169:100–109.
93. Chambers TJ, McCourt DW, Rice CM. Production of yellow fever virus proteins in infected cells: Identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. *Virology* 1990;177:159–174.
94. Chambers TJ, Nestorowicz A, Amberg SM, Rice CM. Mutagenesis of the yellow fever virus NS2B protein: Effects on proteolytic processing, NS2B-NS3 complex formation, and viral replication. *J Virol* 1993;67:6797–6807.
95. Chambers TJ, Nestorowicz A, Mason PW, Rice CM. Yellow fever/Japanese encephalitis chimeric viruses: Construction and biological properties. *J Virol* 1999;73:3095–3101.
96. Chambers TJ, Nestorowicz A, Rice CM. Mutagenesis of the yellow fever virus NS2B/3 cleavage site: Determinants of cleavage site specificity and effects on polyprotein processing and viral replication. *J Virol* 1995;69:1600–1605.
97. Chambers TJ, Weir RC, Grakoui A, et al. Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. *Proc Natl Acad Sci U S A* 1990;87:8898–8902.
98. Chang J, Yang S-H, Cho Y-G, et al. Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-*ras* oncogene. *J Virol* 1998;72:3060–3065.
99. Chang SC, Yen J-H, Kang H-Y, et al. Nuclear localization signals in the core protein of hepatitis C virus. *Biochem Biophys Res Comm* 1994;205:1284–1290.
100. Charrel RN, De Micco P, de Lamballerie X. Phylogenetic analysis of GB viruses A and C: Evidence for cospeciation between virus isolates and their primate hosts. *J Gen Virol* 1999;80:2329–2335.
101. Chayama K, Tsubota A, Koida I, et al. Nucleotide sequence of hepatitis C virus (type 3b) isolated from a Japanese patient with chronic hepatitis C. *J Gen Virol* 1994;75:3623–3628.
102. Chen C-M, You L-R, Hwang L-H, Lee Y-H. Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-b receptor. *J Virol* 1997;71:9417–9426.
103. Chen CJ, Kuo MD, Chien LJ, et al. RNA-protein interactions: Involvement of NS3, NS5, and 3' noncoding regions of Japanese encephalitis virus genomic RNA. *J Virol* 1997;71:3466–3473.
104. Chen LK, Liao CL, Lin CG, et al. Persistence of Japanese encephalitis virus is associated with abnormal expression of the nonstructural protein NS1 in host cells. *Virology* 1996;217:220–229.
105. Chen M, Sonnerborg A, Johansson B, Sallberg M. Detection of hepatitis G virus (GB virus C) RNA in human saliva. *J Clin Microbiol* 1997;35:973–975.
106. Chen P-J, Lin M-H, Tai K-F, et al. The Taiwanese hepatitis C virus genome: Sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. *Virology* 1992;188:102–113.
107. Chen Y, Maguire T, Hileman RE, et al. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate [see comments]. *Nat Med* 1997;3:866–871.
108. Cheng JC, Chang MF, Chang SC. Specific interaction between the hepatitis C virus NS5B RNA polymerase and the 3' end of the viral RNA. *J Virol* 1999;73:7044–7049.
109. Chisari FV. Hepatitis B virus transgenic mice: Models of viral

immunobiology and pathogenesis. *Curr Top Microbiol Immunol* 1996; 206:149–173.
110. Cho HS, Ha NC, Kang LW, et al. Crystal structure of RNA helicase from genotype 1b hepatitis C virus. A feasible mechanism of unwinding duplex RNA. *J Biol Chem* 1998;273:15045–15052.
111. Chon SK, Perez DR, Donis RO. Genetic analysis of the internal ribosome entry segment of bovine viral diarrhea virus. *Virology* 1998;251: 370–382.
112. Choo Q-L, Kuo G, Ralston R, et al. Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc Natl Acad Sci U S A* 1994;91:1294–1298.
113. Choo Q-L, Kuo G, Weiner AJ, et al. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989;244:359–362.
114. Chookhi A, Ung S, Wychowski C, Dubuisson J. Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. *J Virol* 1998;72:3851–3858.
115. Chu PW, Westaway EG. Characterization of Kunjin virus RNA-dependent RNA polymerase: Reinitiation of synthesis *in vitro*. *Virology* 1987;157:330–337.
116. Chu PW, Westaway EG. Molecular and ultrastructural analysis of heavy membrane fractions associated with the replication of Kunjin virus RNA. *Arch Virol* 1992;125:177–191.
117. Chu PWG, Westaway EG. Replication strategy of Kunjin virus: Evidence for recycling role of replicative form RNA as template in semiconservative and asymmetric replication. *Virology* 1985;140:68–79.
118. Chung KM, Lee J, Kim JE, et al. Nonstructural protein 5A of hepatitis C virus inhibits the function of karyopherin beta3. *J Virol* 2000;74:5233–5241.
119. Chung KM, Song OK, Jang SK. Hepatitis C virus nonstructural protein 5A contains potential transcriptional activator domains. *Mol Cells* 1997;7:661–667.
120. Cleaves GR, Ryan TE, Schlesinger RW. Identification and characterization of type 2 dengue virus replicative intermediate and replicative form RNAs. *Virology* 1981;111:73–83.
121. Clum S, Ebner KE, Padmanabhan R. Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient *in vitro* processing and is mediated through the hydrophobic regions of NS2B. *J Biol Chem* 1997; 272:30715–30723.
122. Cocquerel L, Duvet S, Meunier J-C, et al. The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. *J Virol* 1999;73:2641–2649.
123. Cocquerel L, Meunier J-C, Pillez A, et al. A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. *J Virol* 1998;72:2183–2191.
124. Collett MS, Anderson DK, Retzel E. Comparisons of the pestivirus bovine viral diarrhoca virus with members of the flaviviridae. *J Gen Virol* 1988;69:2637–2643.
125. Collett MS, Larson R, Belzer SK, Retzel E. Proteins encoded by bovine viral diarrhea virus: The genomic organization of a pestivirus. *Virology* 1988;165:200–208.
126. Collett MS, Larson R, Gold C, et al. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virology* 1988; 165:191–199.
127. Collett MS, Wiskerchen MA, Welniak E, Belzer SK. Bovine viral diarrhea virus genomic organization. *Arch Virol Suppl* 1991;3:19–27.
128. Colombage G, Hall R, Pavy M, Lobigs M. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. *Virology* 1998;250:151–163.
129. Colombatto P, Randone A, Civitico G, et al. Hepatitis G virus RNA in the serum of patients with elevated gamma glutamyl transpeptidase and alkaline phosphatase: A specific liver disease? [corrected] [published erratum appears in *J Viral Hepat* 1997;4:143]. *J Viral Hepat* 1996;3:301–306.
130. Corapi WV, French TW, Dubovi EJ. Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhea virus. *J Virol* 1989;63:3934–3943.
131. Cortese VS, Grooms DL, Ellis J, et al. Protection of pregnant cattle and their fetuses against infection with bovine viral diarrhea virus type 1 by use of a modified-live virus vaccine. *Am J Vet Res* 1998;59: 1409–1413.
132. Crooks AJ, Lee JM, Easterbrook LM, et al. The NS1 protein of tick-borne encephalitis virus forms multimeric species upon secretion from the host cell. *J Gen Virol* 1994;75:3453–3460.
133. Cui T, Sugrue RJ, Xu Q, et al. Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. *Virology* 1998;246:409–417.
134. Dash S, Halim A-B, Tsuji H, et al. Transfection of HepG2 cells with infectious hepatitis C virus genome. *Am J Pathol* 1997;151:363–373.
135. De Francesco R, Urbani A, Nardi MC, et al. A zinc binding site in viral serine proteinases. *Biochemistry* 1996;35:13282–13287.
136. De Moerlooze L, Desport M, Renard A, et al. The coding region for the 54-kd protein of several pestiviruses lacks host insertions but reveals a "zinc finger-like" domain. *Virology* 1990;177:812–815.
137. De Moerlooze L, Lecomte C, Brown-Shimmer S, et al. Nucleotide sequence of the bovine viral diarrhoea virus Osloss strain: Comparison with related viruses and identification of specific DNA probes in the 5′ untranslated region. *J Gen Virol* 1993;74:1433–1438.
138. Deleersnyder V, Pillez A, Wychowski C, et al. Formation of native hepatitis C virus glycoprotein complexes. *J Virol* 1997;71:697–704.
139. Deng R, Brock KV. Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. *Virology* 1992;191:867–869.
140. Deng R, Brock KV. 5′ and 3′ untranslated regions of pestivirus genome: Primary and secondary structure analyses. *Nucleic Acids Res* 1993;21:1949–1957.
141. Der SD, Yang YL, Weissmann C, Williams BR. A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. *Proc Natl Acad Sci U S A* 1997;94:3279–3283.
142. Deubel V, Digoutte J-P, Mattei X, Pandare D. Morphogenesis of yellow fever virus in *Aedes aegypti* cultured cells. II. An ultrastructural study. *Am J Trop Med Hyg* 1981;30:1071–1077.
143. Dille BJ, Surowy TK, Gutierrez RA, et al. An ELISA for detection of antibodies to the E2 protein of GB virus C. *J Infect Dis* 1997;175: 458–461.
144. Dimasi N, Pasquo A, Martin F, et al. Engineering, characterization and phage display of hepatitis C virus NS3 protease and NS4A cofactor peptide as a single-chain protein. *Protein Eng* 1998;11:1257–1265.
145. Donis RO, Corapi W, Dubovi EJ. Neutralizing monoclonal antibodies to bovine viral diarrhoea virus bind to the 56K to 58K glycoprotein. *J Gen Virol* 1988;69:77–86.
146. Droll DA, Krishna Murthy HM, Chambers TJ. Yellow fever virus NS2B-3 protease: Charged-to-alanine mutagenesis and deletion analysis define regions important for protease complex formation and function. *Virology* 2000;275:33–47.
147. Dubuisson J, Duvet S, Meunier JC, et al. Glycosylation of the hepatitis C virus envelope protein E1 is dependent on the presence of a downstream sequence on the viral polyprotein. *J Biol Chem* 2000; 275:30605–30609.
148. Dubuisson J, Hsu HH, Cheung RC, et al. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J Virol* 1994;68:6147–6160.
149. Dubuisson J, Rice CM. Hepatitis C virus glycoprotein folding: Disulfide bond formation and association with calnexin. *J Virol* 1996;70: 778–786.
150. Duvet S, Cocquerel L, Pillez A, et al. Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. *J Biol Chem* 1998;273: 32088–32095.
151. Eastman PS, Blair CD. Temperature-sensitive mutants of Japanese encephalitis virus. *J Virol* 1985;55:611–616.
152. Eckart MR, Selby M, Masiarz F, et al. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. *Biochem Biophys Res Commun* 1993;192:399–406.
153. Ecker M, Allison SL, Meixner T, Heinz FX. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *J Gen Virol* 1999;80:179–185.
154. Elbers K, Tautz N, Becher P, et al. Processing in the pestivirus E2-NS2 region: Identification of proteins p7 and E2p7. *J Virol* 1996;70: 4131–4135.
155. Enomoto N, Sakuma I, Asahina Y, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. *J Clin Invest* 1995;96:224–230.

156. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996;334:77–81.
157. Failla C, Tomei L, DeFrancesco R. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J Virol* 1994;68:3753–3760.
158. Failla C, Tomei L, DeFrancesco R. An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. *J Virol* 1995;69:1769–1777.
159. Falgout B, Bray M, Schlesinger JJ, Lai CJ. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. *J Virol* 1990,64:4356–4363.
160. Falgout B, Chanock R, Lai C-J. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. *J Virol* 1989;63:1852–1860.
161. Falgout B, Markoff L. Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. *J Virol* 1995;69:7232–7243.
162. Falgout B, Miller RH, Lai C-J. Deletion analysis of Dengue virus type 4 nonstructural protein NS2B: Identification of a domain required for NS2B-NS3 proteinase activity. *J Virol* 1993;67:2034–2042.
163. Falgout B, Pethel M, Zhang Y-M, Lai C-J. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of Dengue virus nonstructural proteins. *J Virol* 1991;65:2467–2475.
164. Farci P, Alter HJ, Wong DC, et al. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated *in vitro* neutralization. *Proc Natl Acad Sci U S A* 1994;91:7792–7796.
165. Farci P, Shimoda A, Wong D, et al. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A* 1996;93:15394–15399.
166. Feinstone SM, Mihalik KB, Kamimura T, et al. Inactivation of hepatitis B virus and non-A, non-B hepatitis by chloroform. *Infect Immun* 1983;41:816–821.
167. Ferrari E, Wright-Minogue J, Fang JW, et al. Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. *J Virol* 1999;73:1649–1654.
168. Fiordalisi G, Zanella I, Mantero G, et al. High prevalence of GB virus C infection in a group of Italian patients with hepatitis of unknown etiology. *J Infect Dis* 1996;174:181–183.
169. Fischler B, Lara C, Chen M, et al. Genetic evidence for mother-to-infant transmission of hepatitis G virus. *J Infect Dis* 1997;176:281–285.
170. Flamand M, Megret F, Mathieu M, et al. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol* 1999; 73:6104–6110.
171. Flint M, Maidens C, Loomis-Price LD, et al. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J Virol* 1999;73:6235–6244.
172. Flint M, McKeating JA. The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. *J Gen Virol* 1999;80:1943–1947.
173. Flint M, Thomas JM, Maidens CM, et al. Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. *J Virol* 1999;73: 6782–6790.
174. Flores EF, Kreutz LC, Donis RO. Swine and ruminant pestiviruses require the same cellular factor to enter bovine cells. *J Gen Virol* 1996; 77:1295–1303.
175. Forns X, Thimme R, Govindarajan S, et al. Hepatitis C virus lacking the hypervariable region 1 of the second envelope glycoprotein is infectious and causes acute resolving or persistent infection in chimpanzees. *Proc Natl Acad Sci U S A* 2000;97:13318–13323.
176. Forwood JK, Brooks A, Briggs LJ, et al. The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site. *Biochem Biophys Res Commun* 1999;257: 731–737.
177. Francki RIB, Fauquet CM, Knudson DL, Brown F. Classification and nomenclature of viruses: Fifth report of the international committee on taxonomy of viruses. *Arch Virol Suppl* 1991;2:223.
178. Francois C, Duverlie G, Rebouillat D, et al. Expression of hepatitis C virus proteins interferes with the antiviral action of interferon independently of PKR-mediated control of protein synthesis. *J Virol* 2000; 74:5587–5596.
179. Frolov I, McBride MS, Rice CM. cis-acting RNA elements required for replication of bovine viral diarrhea virus-hepatitis C virus 5′ nontranslated region chimeras. *RNA* 1998;4:1418–1435.
180. Fujita T, Ishida S, Muramatsu S, et al. Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus. *Biochem Biophys Res Commun* 1996;229:825–831.
181. Fukuma T, Enomoto N, Marumo F, Sato C. Mutations in the interferon-sensitivity determining region of hepatitis C virus and transcriptional activity of the nonstructural region 5A protein. *Hepatology* 1998;28:1147–1153.
182. Fukushi S, Katayama K, Kurihara C, et al. Complete 5′ noncoding region is necessary for the efficient internal initiation of hepatitis C virus RNA. *Biochem Biophys Res Commun* 1994;199:425–432.
183. Fukushi S, Kurihara C, Ishiyama N, et al. Nucleotide sequence of the 5′ noncoding region of hepatitis G virus isolated from Japanese patients: Comparison with reported isolates. *Biochem Biophys Res Commun* 1996;226:314–318.
184. Fukushi S, Kurihara C, Ishiyama N, et al. The sequence element of the internal ribosome entry site and a 25-kilodalton cellular protein contribute to efficient internal initiation of translation of hepatitis C virus RNA. *J Virol* 1997;71:1662–1666.
185. Gale M Jr, Blakely CM, Kwieciszewski B, et al. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: Molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998;18:5208–5218.
186. Gale M Jr, Kwieciszewski B, Dossett M, et al. Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. *J Virol* 1999;73: 6506–6516.
187. Gale MJ Jr, Korth MJ, Tang NM, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 1997;230. 217–227.
188. Gallinari P, Brennan D, Nardi C, et al. Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. *J Virol* 1998;72:6758–6769.
189. Galun E, Burakova T, Ketzinel M, et al. Hepatitis C virus viremia in SCID→BNX mouse chimera. *J Infect Dis* 1995;172:25–30.
190. Ghosh AK, Majumder M, Steele R, et al. Hepatitis C virus NS5A protein modulates transcription through a novel cellular transcription factor SRCAP. *J Biol Chem* 2000;275:7184–7188.
191. Ghosh AK, Steele R, Meyer K, et al. Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth. *J Gen Virol* 1999;80:1179–1183.
192. Gollins SW, Porterfield JS. Flavivirus infection enhancement in macrophages: An electron microscopic study of viral cellular entry. *J Gen Virol* 1985;66:1969–1982.
193. Gollins SW, Porterfield JS. pH-dependent fusion between the flavivirus West Nile and liposomal model membranes. *J Gen Virol* 1986;67:157–166.
194. Gollins SW, Porterfield JS. The uncoating and infectivity of the flavivirus West Nile on interaction with cells: Effects of pH and ammonium chloride. *J Gen Virol* 1986;67:1941–1950
195. Gong Y, Shannon A, Westaway EG, Gowans EJ. The replicative intermediate molecule of bovine viral diarrhoea virus contains multiple nascent strands. *Arch Virol* 1998;143:399–404.
196. Gong Y, Trowbridge R, Macnaughton TB, et al. Characterization of RNA synthesis during a one-step growth curve and of the replication mechanism of bovine viral diarrhoea virus. *J Gen Virol* 1996; 2729–2736.
197. Gontarek RR, Gutshall LL, Herold KM, et al. hnRNP *C* and polypyrimidine tract-binding protein specifically interact with the pyrimidine-rich region within the 3′NTR of the HCV RNA genome. *Nucleic Acids Res* 1999;27:1457–1463.
198. Gorbalenya AE, Donchenko AP, Koonin EV, Blinov VM. N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. *Nucleic Acids Res* 1989;17:3889–3897.
199. Gorbalenya AF, Koonin EV, Donchenko AP, Blinov VM. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res* 1989;17:4713–4729.
200. Gorbalenya AE, Snijder EJ. Viral cysteine proteinases. *Perspect Drug Disc Des* 1996;6:64–86.
201. Grace K, Gartland M, Karayiannis P, et al. The 5′ untranslated region of GB virus B shows functional similarity to the internal ribosome entry site of hepatitis C virus. *J Gen Virol* 1999;80:2337–2341.

202. Grakoui A, Hanson HL, Rice CM. Bad time for Bonzo? Experimental models of HCV infection, replication and pathogenesis. *Hepatology* 2001 (in press).
203. Grakoui A, McCourt DW, Wychowski C, et al. Characterization of the hepatitis C virus-encoded serine proteinase: Determination of proteinase-dependent polyprotein cleavage sites. *J Virol* 1993;67: 2832–2843.
204. Grakoui A, McCourt DW, Wychowski C, et al. A second hepatitis C virus-encoded proteinase. *Proc Natl Acad Sci U S A* 1993;90: 10583–10587.
205. Grakoui A, Wychowski C, Lin C, et al. Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* 1993;67: 1385–1395.
206. Grange T, Bouloy M, Girard M. Stable secondary structure at the 3′ end of the genome of yellow fever virus (17D vaccine strain). *FEBS Lett* 1985;188:159–163.
207. Grassmann CW, Isken O, Behrens SE. Assignment of the multifunctional NS3 protein of bovine viral diarrhea virus during RNA replication: An *in vivo* and *in vitro* study. *J Virol* 1999;73:9196–9205.
208. Gray EW, Nettleton PF. The ultrastructure of cell cultures infected with border discase and bovine virus diarrhoea viruses. *J Gen Virol* 1987;68:2339–2346.
209. Greiser-Wilke I, Dittmar KE, Liess B, Moennig V. Immunofluorescence studies of biotype-specific expression of bovine viral diarrhoea virus epitopes in infected cells. *J Gen Virol* 1991;72:2015–2019.
210. Greiser-Wilke I, Dittmar KE, Liess B, Moennig V. Heterogeneous expression of the non-structural protein p80/p125 in cells infected with different pestiviruses. *J Gen Virol* 1992;73:47–52.
211. Greiser-Wilke I, Haas L, Dittmar K, et al. RNA insertions and gene duplications in the nonstructural protein p125 region of pestivirus strains and isolates *in vitro* and *in vivo*. *Virology* 1992;193:977–980.
212. Grief C, Galler R, Cortes LM, Barth OM. Intracellular localisation of dengue-2 RNA in mosquito cell culture using electron microscopic in situ hybridisation. *Arch Virol* 1997;142:2347–2357.
213. Gritsun TS, Gould EA. Infectious transcripts of tick-borne encephalitis virus, generated in days by RT-PCR. *Virology* 1995;214:611–618.
214. Grun JB, Brinton MA. Characterization of West Nile virus RNA-dependent RNA polymerase and cellular terminal adenylyl and uridylyl transferase in cell-free extracts. *J Virol* 1986;60:1113–1124.
215. Grun JB, Brinton MA. Dissociation of NS5 from cell fractions containing West Nile virus-specific polymerase activity. *J Virol* 1987;61: 3641–3644.
216. Grun JB, Brinton MA. Separation of functional West Nile virus replication complexes from intracellular membrane fragments. *J Gen Virol* 1988;69:3121–3127.
217. Gu B, Liu C, Lin-Goerke J, et al. The RNA helicase and nucleotide triphosphatase activities of the bovine viral diarrhea virus NS3 protein are essential for viral replication. *J Virol* 2000;74:1794–1800.
218. Gualano RC, Pryor MJ, Cauchi MR, et al. Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. *J Gen Virol* 1998;79:437–446.
219. Gubler DJ, Meltzer M. Impact of dengue/dengue hemorrhagic fever on the developing world. *Adv Virus Res* 1999;53:35–70.
220. Guirakhoo F, Bolin RA, Roehrig JT. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. *Virology* 1992;191:921–931.
221. Guirakhoo F, Heinz FX, Kunz C. Epitope model of tick-borne encephalitis virus envelope glycoprotein E: Analysis of structural properties, role of carbohydrate side chain, and conformational changes occurring at acidic pH. *Virology* 1989;169:90–99.
222. Guirakhoo F, Heinz FX, Mandl CW, et al. Fusion activity of flaviviruses: Comparison of mature and immature (prM-containing) tick-borne encephalitis virions. *J Gen Virol* 1991;72:1323–1329.
223. Guirakhoo F, Weltzin R, Chambers TJ, et al. Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. *J Virol* 2000;74:5477–5485.
224. Guirakhoo F, Zhang ZX, Chambers TJ, et al. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. *Virology* 1999; 257:363–372.
225. Gunji T, Kato N, Hijikata M, et al. Specific detection of positive and negative stranded hepatitis C viral RNA using chemical RNA modification. *Arch Virol* 1994;134:293–302.
226. Gutierrez RA, Dawson GJ, Knigge MF, et al. Seroprevalence of GB virus C and persistence of RNA and antibody. *J Med Virol* 1997;53:167–173.
227. Gwack Y, Kim DW, Han JH, Choe J. Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochem Biophys Res Commun* 1996;225:654–659.
228. Gwack Y, Kim DW, Han JH, Choe J. DNA helicase activity of the hepatitis C virus nonstructural protein 3. *Eur J Biochem* 1997;250: 47–54.
229. Gwack Y, Yoo H, Song I, et al. RNA-stimulated ATPase and RNA helicase activities and RNA binding domain of hepatitis G virus nonstructural protein 3. *J Virol* 1999;73:2909–2915.
230. Hahm B, Kim YK, Kim JH, et al. Heterogeneous nuclear ribonucleoprotein L interacts with the 3′ border of the internal ribosomal entry site of hepatitis C virus. *J Virol* 1998;72:8782–8788.
231. Hahn CS, Hahn YS, Rice CM, et al. Conserved elements in the 3′ untranslated region of flavivirus RNAs and potential cyclization sequences. *J Mol Biol* 1987;198:33–41.
232. Hahn YS, Galler R, Hunkapiller T, et al. Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* 1988;162:167–180.
233. Hall RA, Khromykh AA, Mackenzie JM, et al. Loss of dimerisation of the nonstructural protein NS1 of Kunjin virus delays viral replication and reduces virulence in mice, but still allows secretion of NS1. *Virology* 1999;264:66–75.
234. Halstead SB. Pathogenesis of dengue: Challenges to molecular biology. *Science* 1988;239:476–481.
235. Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: A pathogenetic cascade. *Rev Infect Dis* 1989;11 (suppl 4):S830–839.
236. Halstead SB, O'Rourke EJ. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 1977;265:739–741.
237. Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J Exp Med* 1977;146:201–217.
238. Halstead SB, O'Rourke EJ, Allison AC. Dengue viruses and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting *in vitro* infection. *J Exp Med* 1977;146:218–229.
239. Han JH, Shyamala V, Richman KH, et al. Characterization of the terminal regions of hepatitis C viral RNA: Identification of conserved sequences in the 5′ untranslated region and poly(A) tails at the 3′ end. *Proc Natl Acad Sci U S A* 1991;88:1711–1715.
240. Handa A, Jubran RF, Dickstein B, et al. GB virus C/hepatitis G virus infection is frequent in American children and young adults. *Clin Infect Dis* 2000;30:569–571.
241. Harada S, Watanabe Y, Takeuchi K, et al. Expression of processed core protein of hepatitis C virus in mammalian cells. *J Virol* 1991;65: 3015–3021.
242. Harada T, Tautz N, Thiel HJ. E2-p7 region of the bovine viral diarrhea virus polyprotein: Processing and functional studies. *J Virol* 2000;74: 9498–9506.
243. Harpin S, Hurley DJ, Mbikay M, et al. Vaccination of cattle with a DNA plasmid encoding the bovine viral diarrhoea virus major glycoprotein E2. *J Gen Virol* 1999;80:3137–3144.
244. Hase T, Summers PL, Cohen WH. A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis viruses. *Arch Virol* 1989;108:101–114.
245. Hase T, Summers PL, Eckels KH. Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch Virol* 1989; 104:129–143.
246. Hase T, Summers PL, Eckels KH, Baze WB. An electron and immunocletron microscopic study of dengue-2 virus infection of cultured mosquito cells: Maturation events. *Arch Virol* 1987;92:273–291.
247. Hase T, Summers PL, Eckels KH, Baze WB. Maturation process of Japanese encephalitis virus in cultured mosquito cells *in vitro* and mouse brain cells *in vivo*. *Arch Virol* 1987;96:135–151.
248. Hashimoto H, Nomoto A, Watanabe K, et al. Molecular cloning and complete nucleotide sequence of the genome of Japanese encephalitis virus Beijing-1 strain. *Virus Genes* 1988;1:305–317.
249. Hayashi N, Higashi H, Kaminaka K, et al. Molecular cloning and heterogeneity of the human hepatitis C virus (HCV) genome. *J Hepatol* 1993;17(suppl. 3):S94–107.
250. He LF, Alling D, Popkin T, et al. Determining the size of non-A, non-B hepatitis by filtration. *J Infect Dis* 1987;156:636–640.
251. He RT, Innis BL, Nisalak A, et al. Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing

antibody response against dengue virus type 2. *J Med Virol* 1995;45: 451–461.
252. Heilek GM, Peterson MG. A point mutation abolishes the helicase but not the nucleoside triphosphatase activity of hepatitis C virus NS3 protein. *J Virol* 1997;71:6264–6266.
253. Heinz FX. Epitope mapping of flavivirus glycoproteins. *Adv Virus Res* 1986;31:103–168.
254. Heinz FX, Stiasny K, Puschner-Auer G, et al. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. *Virology* 1994; 198:109–117.
255. Henchal EA, Henchal LS, Schlesinger JJ. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. *J Gen Virol* 1988;69: 2101–2107.
256. Higginbottom A, Quinn ER, Kuo CC, et al. Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. *J Virol* 2000;74:3642–3649.
257. Hijikata M, Kato N, Ootsuyama Y, et al. Hypervariable regions in the putative glycoprotein of hepatitis C virus. *Biochem Biophys Res Commun* 1991;175:220–228.
258. Hijikata M, Kato N, Ootsuyama Y, et al. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. *Proc Natl Acad Sci U S A* 1991;88:5547–5551.
259. Hijikata M, Mizushima H, Akagi T, et al. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol* 1993;67:4665–4675.
260. Hijikata M, Mizushima H, Tanji Y, et al. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc Natl Acad Sci U S A* 1993;90:10773–10777.
261. Hijikata M, Shimizu YK, Kato H, et al. Equilibrium centrifugation studies of hepatitis C virus: Evidence for circulating immune complexes. *J Virol* 1993;67:1953–1958.
262. Hirota M, Satoh S, Asabe S, et al. Phosphorylation of nonstructural 5A protein of hepatitis C virus: HCV group-specific hyperphosphorylation. *Virology* 1999;257:130–137.
263. Hirowatari Y, Hijikata M, Tanji Y, et al. Two proteinase activities in HCV polypeptide expressed in insect cells using baculovirus vector. *Arch Virol* 1993;133:349–356.
264. Hoff HS, Donis RO. Induction of apoptosis and cleavage of poly (ADP-ribose) polymerase by cytopathic bovine viral diarrhea virus infection. *Virus Res* 1997;49:101–113.
265. Hollinger FB, Gitnick G, Aach RD, et al. Non-A, non-B hepatitis transmission in chimpanzees: A project of the transfusion-transmitted viruses study group. *Intervirology* 1978;10:60–68.
266. Hollingshead PG, Brawner TA, Fleming TP. St. Louis encephalitis virus temperature-sensitive mutants. I. Induction, isolation, and preliminary characterization. *Arch Virol* 1983;75:171–179.
267. Honda A, Arai Y, Hirota N, et al. Hepatitis C virus structural proteins induce liver cell injury in transgenic mice. *J Med Virol* 1999;59:281–289.
268. Honda M, Brown EA, Lemon SM. Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. *RNA* 1996;2:955–968.
269. Honda M, Kaneko S, Matsushita E, et al. Cell cycle regulation of hepatitis C virus internal ribosome entry site-directed translation. *Gastroenterology* 2000;118:152–162.
270. Honda M, Ping LH, Rijnbrand RC, et al. Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. *Virology* 1996;222:31–42.
271. Hong Z, Perrari E, Wright-Minogue J, et al. Enzymatic characterization of hepatitis C virus NS3/4A complexes expressed in mammalian cells by using the herpes simplex virus amplicon system. *J Virol* 1996; 70:4261–4268.
272. Hori H, Lai C-J. Cleavage of dengue virus NS1-NS2A requires an octapeptide sequence at the C terminus of NS1. *J Virol* 1990;64: 4572–4577.
273. Horzinek MC. *Non-Arthropod-Borne Togaviruses*. London: Academic Press, 1981.
274. Howe AY, Chase R, Taremi SS, et al. A novel recombinant single-chain hepatitis C virus NS3-NS4A protein with improved helicase activity. *Protein Sci* 1999;8:1332–1341.
275. Hsu HH, Donets M, Greenberg HB, Feinstone SM. Characterization of hepatitis C virus structural proteins with a recombinant baculovirus expression system. *Hepatology* 1993;17:763–771.
276. Hulst MM, Himes G, Newbigin E, Moorman RJM. Glycoprotein E2 of classical swine fever virus: Expression in insect cells and identification as a ribonuclease. *Virology* 1994;200:558–565.
277. Hulst MM, Moormann RJ. Inhibition of pestivirus infection in cell culture by envelope proteins E(rns) and E2 of classical swine fever virus: E(rns) and E2 interact with different receptors. *J Gen Virol* 1997;78:2779–2787.
278. Hulst MM, Panoto FE, Hoekman A, et al. Inactivation of the RNase activity of glycoprotein E^{rns} results in a cytopathogenic virus. *J Virol* 1998;72:151–157.
279. Hulst MM, Westra DF, Wensvoort G, Moormann RJ. Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. *J Virol* 1993;67:5435–5442.
280. Hung SL, Lee PL, Chen HW, et al. Analysis of the steps involved in Dengue virus entry into host cells. *Virology* 1999;257:156–167.
281. Hurrelbrink RJ, Nestorowicz A, McMinn PC. Characterization of infectious Murray Valley encephalitis virus derived from a stably cloned genome-length cDNA. *J Gen Virol* 1999;80:3115–3125.
282. Hüssy P, Langen H, Mous J, Jacobsen H. Hepatitis C virus core protein: Carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. *Virology* 1996;224:93–104.
283. Hüssy P, Schmid G, Mous J, Jacobsen H. Purification and *in vitro*-phospholabeling of secretory envelope proteins E1 and E2 of hepatitis C virus expressed in insect cells. *Virus Res* 1996;45: 45–57.
284. Hwang SB, Lo S-Y, Ou J-H, Lai MMC. Detection of cellular proteins and viral core protein interacting with the 5′ untranslated region of hepatitis C virus RNA. *J Biomed Sci* 1995;2:227–236.
285. Ide Y, Tanimoto A, Sasaguri Y, Padmanabhan R. Hepatitis C virus NS5A protein is phosphorylated *in vitro* by a stably bound protein kinase from HeLa cells and by cAMP-dependent protein kinase A-a catalytic subunit. *Gene* 1997;201:151–158.
286. Ide Y, Zhang L, Chen M, et al. Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. *Gene* 1996;182:203–211.
287. Iizuka N, Najita L, Franzusoff A, Sarnow P. Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. *Mol Cell Biol* 1994; 14:7322–7330.
288. Ikeda M, Sugiyama K, Tanaka T, et al. Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. *Biochem Biophys Res Commun* 1998;245:549–553.
289. Inoue Y, Miyazaki M, Ohashi R, et al. Ubiquitous presence of cellular proteins that specifically bind to the 3′ terminal region of hepatitis C virus. *Biochem Biophys Res Commun* 1998;245:198–203.
290. Imideh M, Nyunoya H, Tanaka T, et al. Antigenicity of hepatitis C virus envelope proteins expressed in Chinese hamster ovary cells. *Vaccine* 1996;14:1590–1596.
291. Iqbal M, Flick-Smith H, McCauley JW. Interactions of bovine viral diarrhoea virus glycoprotein E(rns) with cell surface glycosaminoglycans. *J Gen Virol* 2000;81:451–459.
292. Ishak R, Tovey DG, Howard CR. Morphogenesis of yellow fever virus 17D in infected cell cultures. *J Gen Virol* 1988;69:325–335.
293. Ishido S, Fujita T, Hotta H. Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. *Biochem Biophys Res Commun* 1998;244:35–40.
294. Ishido S, Muramatsu S, Fujita T, et al. Wild-type, but not mutant-type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus. *Biochem Biophys Res Commun* 1997;230:431–436.
295. Ito T, Lai MMC. Determination of the secondary structure of and cellular protein binding to the 3′-untranslated region of the hepatitis C virus RNA genome. *J Virol* 1997;71:8698–8706.
296. Ito T, Tahara SM, Lai MMC. The 3′-untranslated region of hepatitis C virus RNA enhances translation from an internal ribosomal entry site. *J Virol* 1998;72:8789–8796.
297. Jacobs SC, Stephenson JR, Wilkinson GW. High-level expression of the tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector. Protection elicited in a murine model. *J Virol* 1992;66: 2086–2095.
298. Jäger J, Smerdon SJ, Wang J, et al. Comparison of three different crystal forms shows HIV-1 reverse transcriptase displays an internal swivel motion. *Structure* 1994;2:869–876.
299. Jan LR, Yang CS, Trent DW, et al. Processing of Japanese encephalitis virus non-structural proteins: NS2B-NS3 complex and heterologous proteases. *J Gen Virol* 1995;76:573–580.
300. Jin DY, Wang HL, Zhou Y, et al. Hepatitis C virus core protein-

induced loss of LZIP function correlates with cellular transformation. *EMBO J* 2000;19:729–740.

301. Jin L, Peterson DL. Expression, isolation, and characterization of the hepatitis C virus ATPase/RNA helicase. *Arch Biochem Biophys* 1995; 323:47–53.
302. Kadare G, Haenni AL. Virus-encoded RNA helicases. *J Virol* 1997;71: 2583–2590.
303. Kaito M, Watanabe S, Tsukiyama-Kohara K, et al. Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* 1994;75:1755–1760.
304. Kaminski A, Hunt SL, Patton JG, Jackson RJ. Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. *RNA* 1995;1:924–938.
305. Kanai A, Tanabe K, Kohara M. Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. *FEBS Lett* 1995;376: 221–224.
306. Kaneko T, Tanji Y, Satoh S, et al. Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. *Biochem Biophys Res Commun* 1994;205:320–326.
307. Kanto T, Hayashi N, Takehara T, et al. Buoyant density of hepatitis C virus recovered from infected hosts: Two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. *Hepatology* 1994;19:296–302.
308. Kao CC, Del Vecchio AM, Zhong W. De novo initiation of RNA synthesis by a recombinant flaviviridae RNA-dependent RNA polymerase. *Virology* 1999;253:1–7.
309. Kao JH, Chen PJ, Hsiang SC, et al. Phylogenetic analysis of GB virus C: Comparison of isolates from Africa, North America, and Taiwan. *J Infect Dis* 1996;174:410–413.
310. Kapoor M, Zhang L, Mohan PM, Padmanabhan R. Synthesis and characterization of an infectious dengue virus type-2 RNA genome (New Guinea C strain). *Gene* 1995;162:175–180.
311. Kapoor M, Zhang L, Ramachandra M, et al. Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. *J Biol Chem* 1995; 270:19100–19106.
312. Kato N, Hijikata M, Ootsuyama Y, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* 1990;87:9524–9528.
313. Kato N, Lan K-H, Ono-Nita SK, et al. Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. *J Virol* 1997;71: 8856–8859.
314. Kato N, Ootsuyama Y, Ohkoshi S, et al. Characterization of hypervariable regions in the putative envelope protein of hepatitis C virus. *Biochem Biophys Res Commun* 1992;189:119–127.
315. Kato N, Sekiya H, Ootsuyama Y, et al. Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J Virol* 1993;67:3923–3930.
316. Kaufman BM, Summers PL, Dubois DR, et al. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. *Am J Trop Med Hyg* 1989;41:576–580.
317. Kawamura T, Furusaka A, Koziel MJ, et al. Transgenic expression of hepatitis C virus structural proteins in the mouse. *Hepatology* 1997; 25:1014–1021.
318. Khromykh AA, Kenney MT, Westaway EG. trans-Complementation of flavivirus RNA polymerase gene NS5 by using Kunjin virus replicon-expressing BHK cells. *J Virol* 1998;72:7270–7279.
319. Khromykh AA, Sedlak PL, Guyatt KJ, et al. Efficient trans-complementation of the flavivirus kunjin NS5 protein but not of the NS1 protein requires its coexpression with other components of the viral replicase. *J Virol* 1999;73:10272–10280.
320. Khromykh AA, Sedlak PL, Westaway EG. trans-Complementation analysis of the flavivirus Kunjin ns5 gene reveals an essential role for translation of its N-terminal half in RNA replication. *J Virol* 1999;73: 9247–9255.
321. Khromykh AA, Sedlak PL, Westaway EG. cis- and trans-acting elements in flavivirus RNA replication. *J Virol* 2000;74:3253–3263.
322. Khromykh AA, Westaway EG. Completion of Kunjin virus RNA sequence and recovery of an infectious RNA transcribed from stably cloned full-length cDNA. *J Virol* 1994;68:4580–4588.
323. Khromykh AA, Westaway EG. Subgenomic replicons of the flavivirus Kunjin: Construction and applications. *J Virol* 1997;71:1497–1505.
324. Kidd VJ. Proteolytic activities that mediate apoptosis. *Annu Rev Physiol* 1998;60:533–573.
325. Kim DW, Gwack Y, Han JH, Choe J. C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem Biophys Res Commun* 1995;215:160–166.
326. Kim DW, Suzuki R, Harada T, et al. Trans-suppression of gene expression by hepatitis C viral core protein. *Jpn J Med Sci Biol* 1994;47: 211–220.
327. Kim JL, Morgenstern KA, Griffith JP, et al. Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: The crystal structure provides insights into the mode of unwinding. *Structure* 1998;6:89–100.
328. Kim JL, Morgenstern KA, Lin C, et al. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* 1996;87:343–355.
329. Kimura T, Kimura-Kuroda J, Nagashima K, Yasui K. Analysis of virus-cell binding characteristics on the determination of Japanese encephalitis virus susceptibility. *Arch Virol* 1994;139:239–251.
330. Kimura T, Ohyama A. Association between the pH-dependent conformational change of West Nile flavivirus E protein and virus-mediated membrane fusion. *J Gen Virol* 1988;69:1247–1254.
331. Kinney RM, Butrapet S, Chang GJ, et al. Construction of infectious cDNA clones for dengue 2 virus: Strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* 1997;230:300–308.
332. Kiyosawa K, Sodeyama T, Tanaka E, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: Analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990;12:671–675.
333. Ko KK, Igarashi A, Fukai K. Electron microscopic observation on *Aedes albopictus* cells infected with dengue viruses. *Arch Virol* 1979; 62:41–52.
334. Koch JO, Bartenschlager R. Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J Virol* 1999;73:7138–7146.
335. Kohara M, Tsukiyama-Kohara K, Maki N, et al. Expression and characterization of glycoprotein gp35 of hepatitis C virus using recombinant vaccinia virus. *J Gen Virol* 1992;73:2313–2318.
336. Koike K, Moriya K, Ishibashi K, et al. Expression of hepatitis C virus envelope proteins in transgenic mice. *J Gen Virol* 1995;76:3031–3038.
337. Kolykhalov AA, Agapov EV, Blight KJ, et al. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 1997; 277:570–574.
338. Kolykhalov AA, Agapov EV, Rice CM. Specificity of the hepatitis C virus serine proteinase: Effects of substitutions at the 3/4A, 4A/4B, 4B/5A, and 5A/5B cleavage sites on polyprotein processing. *J Virol* 1994;68:7525–7533.
339. Kolykhalov AA, Feinstone SM, Rice CM. Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. *J Virol* 1996;70:3363–3371.
340. Kolykhalov AA, Mihalik K, Feinstone SM, Rice CM. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication *in vivo*. *J Virol* 2000;74:2046–2051.
341. Konishi E, Mason PW. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *J Virol* 1993;67:1672–1675.
342. Konishi E, Pincus S, Paoletti E, et al. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 1992;188:714–720.
343. Konishi E, Yamaoka M, Khin Sane W, et al. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. *J Virol* 1998;72:4925–4930.
344. Koonin EV. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. *J Gen Virol* 1993;74:733–740.
345. Koonin EV, Dolja VV. Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences [published erratum appears in *Crit Rev Biochem Mol Biol* 1993;28:546]. *Crit Rev Biochem Mol Biol* 1993;28:375–430.
346. Kopecky J, Grubhoffer L, Kovar V, et al. A putative host cell receptor for tick-borne encephalitis virus identified by anti-idiotypic antibodies and virus affinoblotting. *Intervirology* 1999;42:9–16.
347. Kümmerer BM, Meyers G. Correlation between point mutations in NS2 and the viability and cytopathogenicity of bovine viral diarrhea virus strain Oregon analyzed with an infectious cDNA clone. *J Virol* 2000;74:390–400.

348. Kümmerer BM, Stoll D, Meyers G. Bovine viral diarrhea virus strain Oregon: A novel mechanism for processing of NS2-3 based on point mutations. *J Virol* 1998;72:4127–4138.
349. Kuno G, Chang GJ, Tsuchiya KR, et al. Phylogeny of the genus *Flavivirus*. *J Virol* 1998;72:73–83.
350. Kupfermann H, Thiel H-J, Dubovi EJ, Meyers G. Bovine viral diarrhea virus: Characterization of a cytopathogenic defective interfering particle with two internal deletions. *J Virol* 1996;70:8175–8181.
351. Kuroski M, Enomoto N, Marumo F, Sato C. Rapid sequence variation of the hypervariable region of hepatitis C virus during the course of chronic infection. *Hepatology* 1993;18:1293–1299.
352. Kyono K, Miyashiro M, Taguchi I. Detection of hepatitis C virus helicase activity using the scintillation proximity assay system. *Anal Biochem* 1998;257:120–126.
353. Lagging LM, Meyer K, Owens RJ, Ray R. Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus. *J Virol* 1998;72:3539–3546.
354. Lai C-J, Zhao B, Hori H, Bray M. Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus. *Proc Natl Acad Sci U S A* 1991;88:5139–5143.
355. Lai VC, Kao CC, Ferrari E, et al. Mutational analysis of bovine viral diarrhea virus RNA-dependent RNA polymerase. *J Virol* 1999;73:10129–10136.
356. Lam NP, Neumann AU, Gretch DR, et al. Dose-dependent acute clearance of hepatitis C genotype 1 virus with interferon alfa. *Hepatology* 1997;26:226–231.
357. Lancaster MH, Hodgetts SI, Mackenzie JS, Urosevic N. Characterization of defective viral RNA produced during persistent infection of Vero cells with Murray Valley encephalitis virus. *J Virol* 1998;72:2474–2482.
358. Lanciotti RS, Roehrig JT, Deubel V, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 1999;286:2333–2337.
359. Lanford RE, Chavez D, Chisari FV, Sureau C. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J Virol* 1995;69:8079–8083.
360. Lanford RE, Notvall L, Chavez D, et al. Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. *Virology* 1993;197:225–235.
361. Laude H. Improved method for the purification of hog cholera virus. *Arch Virol* 1977;54:41–51.
362. Laude H. Hog cholera virus: Art and facts. *Ann Rech Vet* 1987;18:127–138.
363. Leary K, Blair CD. Sequential events in the morphogenesis of Japanese encephalitis virus. *J Ultrastruct Res* 1980;72:123–129.
364. Leary TP, Desai SM, Yamaguchi J, et al. Species-specific variants of GB virus A in captive monkeys [published erratum appears in *J Virol* 1997;71:8953]. *J Virol* 1996;70:9028–9030.
365. Leary TP, Muerhoff AS, Simons JN, et al. Sequence and genomic organization of GBV-C: A novel member of the *Flaviviridae* associated with human non-A-E hepatitis. *J Med Virol* 1996;48:60–67.
366. Lee E, Stocks CE, Amberg SM, et al. Mutagenesis of the signal sequence of yellow fever virus prM protein: Enhancement of signalase cleavage *in vitro* is lethal for virus production. *J Virol* 2000;74:24–32.
367. Lee JM, Crooks AJ, Stephenson JR. The synthesis and maturation of a non-structural extracellular antigen from tick-borne encephalitis virus and its relationship to the intracellular NS1 protein. *J Gen Virol* 1989;70:335–343.
368. Lefrere JJ, Loiseau P, Maury J, et al. Natural history of GB-V-C/hepatitis G virus infection through the follow-up of GBV-C/hepatitis G virus-infected blood donors and recipients studied by RNA polymerase chain reaction and anti-E2 serology. *Blood*. 1997;90:3776–3780.
369. Lefrere JJ, Roudot-Thoraval F, Morand-Joubert L, et al. Prevalence of GB virus type C/hepatitis G virus RNA and of anti-E2 in individuals at high or low risk for blood-borne or sexually transmitted viruses: Evidence of sexual and parenteral transmission. *Transfusion* 1999;39:83–94.
370. Leinbach SS, Bhat RA, Xia S-M, et al. Substrate specificity of the NS3 serine proteinase of hepatitis C virus as determined by mutagenesis at the NS3/NS4A junction. *Virology* 1994;204:163–169.
371. Lesburg CA, Cable MB, Ferrari E, et al. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 1999;6:937–943.
372. Lesniewski R, Okasinski G, Carrick R, et al. Antibody to hepatitis C virus second envelope (HCV-E2) glycoprotein: A new marker of HCV infection closely associated with viremia. *J Med Virol* 1995;45:415–422.
373. Li H, Clum S, You S, et al. The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. *J Virol* 1999;73:3108–3116.
374. Li Y, Koroley S, Waksman G. Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA polymerase I: Structural basis for nucleotide incorporation. *EMBO J* 1998;17:7514–7525.
375. Liebler EM, Waschbusch J, Pohlenz JF, et al. Distribution of antigen of noncytopathogenic and cytopathogenic bovine virus diarrhea virus biotypes in the intestinal tract of calves following experimental production of mucosal disease. *Arch Virol Suppl* 1991;3:109–124.
376. Liess B. Hog cholera. In: *A World Geography of Epidemiology and Control*. London: Academic Press, 1981:627–650.
377. Lin C, Amberg SM, Chambers TJ, Rice CM. Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 protoinase is a prerequisite for processing at the downstream 4A/4B signalase site. *J Virol* 1993;67:2327–2335.
378. Lin C, Chambers TJ, Rice CM. Mutagenesis of conserved residues at the yellow fever virus 3/4A and 4B/5 dibasic cleavage sites: Effects on cleavage efficiency and polyprotein processing. *Virology* 1993;192:596–604.
379. Lin C, Lindenbach BD, Prágai B, et al. Processing of the hepatitis C virus E2-NS2 region: Identification of p7 and two distinct E2-specific products with different C termini. *J Virol* 1994;68:5063–5073.
380. Lin C, Prágai B, Grakoui A, et al. Hepatitis C virus NS3 serine proteinase: Trans-cleavage requirements and processing kinetics. *J Virol* 1994;68:8147–8157.
381. Lin C, Thomson JA, Rice CM. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex *in vivo* and *in vitro*. *J Virol* 1995;69:4373–4380.
382. Lin C, Wu JW, Hsiao K, Su MS. The hepatitis C virus NS4A protein: Interactions with the NS4B and NS5A proteins. *J Virol* 1997;71:6465–6471.
383. Lin YL, Chen LK, Liao CL, et al. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. *J Virol* 1998;72:191–200.
384. Lindenbach BD, Rice CM. trans-Complementation of yellow fever virus NS1 reveals a role in early RNA replication. *J Virol* 1997;71:9608–9617.
385. Lindenbach BD, Rice CM. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J Virol* 1999;73:4611–4621.
386. Linnen J, Wages J, Zhangkeck ZY, et al. Molecular cloning and disease association of hepatitis G virus: A transfusion-transmissible agent. *Science* 1996;271:505–508.
387. Linnen JM, Fung K, Fry KF, et al. Sequence variation and phylogenetic analysis of the 5′ terminus of hepatitis G virus. *J Viral Hepat* 1997;4:293–302.
388. Liprandi F, Walder R. Replication of virulent and attenuated strains of yellow fever virus in human monocytes and macrophage-like cells (U937). *Arch Virol* 1983;76:51–61.
389. Liu Q, Bhat RA, Prince AM, Zhang P. The hepatitis C virus NS2 protein generated by NS2-3 autocleavage is required for NS5A phosphorylation. *Biochem Biophys Res Commun* 1999;254:572–577.
390. Liu Q, Tackney C, Bhat RA, et al. Regulated processing of hepatitis C virus core protein is linked to subcellular localization. *J Virol* 1997;71:657–662.
391. Lo S-Y, Masuzr F, Hwang SB, et al. Differential subcellular localization of hepatitis C virus core gene products. *Virology* 1995;213:455–461.
392. Lo S-Y, Selby M, Tong M, Ou J-H. Comparative studies of the core gene products of two different hepatitis C virus isolates: Two alternative forms determined by a single amino acid substitution. *Virology* 1994;199:124–131.
393. Lo S-Y, Selby MJ, Ou J-H. Interaction between hepatitis C virus core protein and E1 envelope protein. *J Virol* 1996;70:5177–5182.
394. Lobigs M. Proteolytic processing of a Murray Valley encephalitis virus non-structural polyprotein segment containing the viral pro-

teinase: Accumulation of a NS3-4A precursor which requires mature NS3 for efficient processing. *J Gen Virol* 1992;73:2305–2312.
395. Lobigs M. Flavivirus premembrane protein cleavage and spike heterodimer secretion requires the function of the viral proteinase NS3. *Proc Natl Acad Sci U S A* 1993;90:6218–6222.
396. Lohmann V, Körner F, Herian U, Bartenschlager R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol* 1997;71:8416–8428.
397. Lohmann V, Korner F, Koch JO, et al. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999;285: 110–113.
398. Lohmann V, Overton H, Bartenschlager R. Selective stimulation of hepatitis C virus and pestivirus NS5B RNA polymerase activity by GTP. *J Biol Chem* 1999;274:10807–10815.
399. Lohmann V, Roos A, Korner F, et al. Biochemical and kinetic analyses of NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *Virology* 1998;249:108–118.
400. Love RA, Parge H, Wickersham JA, et al. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* 1996;87:331–342.
401. Lu HH, Wimmer E. Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus. *Proc Natl Acad Sci U S A* 1996;93:1412–1417.
402. Luo G, Hamatake RK, Mathis DM, et al. De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. *J Virol* 2000;74:851–863.
403. Mackenzie JM, Jones MK, Westaway EG. Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. *J Virol* 1999;73:9555–9567.
404. Mackenzie JM, Jones MK, Young PR. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. *Virology* 1996;220:232–240.
405. Mackenzie JM, Khromykh AA, Jones MK, Westaway EG. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology* 1998;245:203–215.
406. Major ME, Mihalik K, Fernandez J, et al. Long term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. *J Virol* 1999;73:3317–3325.
407. Manabe S, Fuke I, Tanishita O, et al. Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. *Virology* 1994;198:636–644.
408. Mandl CW, Aberle JH, Aberle SW, et al. *In vitro*-synthesized infectious RNA as an attenuated live vaccine in a flavivirus model [see comments]. *Nat Med* 1998;4:1438–1440.
409. Mandl CW, Allison SL, Holzmann H, et al. Attenuation of tick-borne encephalitis virus by structure-based site-specific mutagenesis of a putative flavivirus receptor binding site. *J Virol* 2000;74:9601–9609.
410. Mandl CW, Ecker M, Holzmann H, et al. Infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr. *J Gen Virol* 1997;78:1049–1057.
411. Mandl CW, Heinz FX, Puchhammer-Stöckl E, Kunz C. Sequencing the termini of capped viral RNA by 5'-3' ligation and PCR. *Biotechniques* 1991;10:486.
412. Mandl CW, Heinz FX, Stöckl E, Kunz C. Genome sequence of tick-borne encephalitis virus (Western subtype) and comparative analysis of nonstructural proteins with other flaviviruses. *Virology* 1989;173: 291–301.
413. Mandl CW, Holzmann H, Kunz C, Heinz FX. Complete genomic sequence of Powassan virus: Evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. *Virology* 1993;194:173–184.
414. Mandl CW, Holzmann H, Meixner T, et al. Spontaneous and engineered deletions in the 3' noncoding region of tick-borne encephalitis virus: Construction of highly attenuated mutants of a flavivirus. *J Virol* 1998;72:2132–2140.
415. Mandl CW, Kunz C, Heinz FX. Presence of poly(A) in a flavivirus: Significant differences between the 3' noncoding regions of the genomic RNAs of tick-borne encephalitis virus strains. *J Virol* 1991; 65:4070–4077.
416. Marianneau P, Megret F, Olivier R, et al. Dengue 1 virus binding to human hepatoma HepG2 and simian Vero cell surfaces differs. *J Gen Virol* 1996;77:2547–2554.
417. Markland W, Petrillo RA, Fitzgibbon M, et al. Purification and characterization of the NS3 serine protease domain of hepatitis C virus expressed in *Saccharomyces cerevisiae*. *J Gen Virol* 1997;78:39–43.
418. Markoff L, Falgout B, Chang A. A conserved internal hydrophobic domain mediates the stable membrane integration of the dengue virus capsid protein. *Virology* 1997;233:105–117.
419. Mason PW. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 1989, 169:354–364.
420. Mason PW, Pincus S, Fournier MJ, et al. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 1991;180:294–305.
421. Masuko K, Mitsui T, Iwano K, et al. Infection with hepatitis GB virus C in patients on maintenance hemodialysis [see comments]. *N Engl J Med* 1996;334:1485–1490.
422. Matsumoto M, Hsieh T-Y, Zhu N, et al. Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-β receptor. *J Virol* 1997;71:1301–1309.
423. Matsumoto M, Hwang SB, Jeng K-S, et al. Homotypic interaction and multimerization of hepatitis C virus core protein. *Virology* 1996;218: 43–51.
424. Matsumura T, Shiraki K, Sashitaka T, Hotta S. Morphogenesis of dengue-1 virus in cultures of a human leukemic leukocyte line (J-111). *Microbiol Immunol* 1977;21:329–334.
425. Matsuura Y, Harada S, Suzuki R, et al. Expression of processed envelope protein of hepatitis C virus in mammalian and insect cells. *J Virol* 1992;66:1425–1431.
426. Matsuura Y, Suzuki T, Suzuki R, et al. Processing of E1 and E2 glycoproteins of hepatitis C virus expressed in mammalian and insect cells. *Virology* 1994;205:141–150.
427. McClurkin AW, Bolin SR, Coria MF. Isolation of cytopathic and noncytopathic bovine viral diarrhea virus from the spleen of cattle acutely and chronically affected with bovine viral diarrhea. *J Am Vet Med Assoc* 1985;186:568–569.
428. Melvin SL, Dawson GJ, Carrick RJ, et al. Biophysical characterization of OB virus C from human plasma. *J Virol Methods* 1998;71:147–157.
429. Men R, Bray M, Clark D, et al. Dengue type 4 virus mutants containing deletion in the 3' noncoding region of the RNA genome: Analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. *J Virol* 1996;70:3930–3937.
430. Mendez E, Ruggli N, Collett MS, Rice CM. Infectious bovine viral diarrhea virus (strain NADL) RNA from stable cDNA clones: A cellular insert determines NS3 production and viral cytopathogenicity. *J Virol* 1998;72:4737–4745.
431. Meola A, Sbardellati A, Bruni Ercole B, et al. Binding of hepatitis C virus E2 glycoprotein to CD81 does not correlate with species permissiveness to infection. *J Virol* 2000;74:5933–5938.
432. Meyers G, Rümenapf T, Thiel H-J. Molecular cloning and nucleotide sequence of the genome of hog cholera virus. *Virology* 1989;171: 555–567.
433. Meyers G, Rümenapf T, Thiel H-J. Ubiquitin in a togavirus [letter]. *Nature* 1989;341:491.
434. Meyers G, Rümenapf T, Thiel H-J. Insertion of ubiquitin-coding sequence in the RNA genome of a togavirus. In: Brinton MA, Heinz FX, eds. *New Aspects of Positive Strand RNA Viruses*. Washington, DC: American Society for Microbiology, 1990:25–29.
435. Meyers G, Saalmuller A, Buttner M. Mutations abrogating the RNase activity in glycoprotein E(rns) of the pestivirus classical swine fever virus lead to virus attenuation. *J Virol* 1999;73:10224–10235.
436. Meyers G, Stoll D, Gunn M. Insertion of a sequence encoding light chain 3 of microtubule-associated proteins 1A and 1B in a pestivirus genome: Connection with virus cytopathogenicity and induction of lethal disease in cattle. *J Virol* 1998;72:4139–4148.
437. Meyers G, Tautz N, Becher P, et al. Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. *J Virol* 1996;70:8606–8613.
438. Meyers G, Tautz N, Dubovi EJ, Thiel H-J. Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. *Virology* 1991, 180:602–616.
439. Meyers G, Tautz N, Stark R, et al. Rearrangement of viral sequences in cytopathogenic pestiviruses. *Virology* 1992;191:368–386.
440. Meyers G, Thiel H-J. Cytopathogenicity of classical swine fever virus caused by defective interfering particles. *J Virol* 1995;69:3683–3689.

441. Meyers G, Thiel H-J. Molecular characterization of pestiviruses. *Adv Virus Res* 1996;47:53–118.

442. Meyers G, Thiel H-J, Rumenapf T. Classical swine fever virus: Recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. *J Virol* 1996;70:1588–1595.

443. Michalak JP, Wychowski C, Choukhi A, et al. Characterization of truncated forms of the hepatitis C virus glycoproteins. *J Gen Virol* 1997;78:2299–2306.

444. Miller RH, Purcell RH. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc Natl Acad Sci U S A* 1990;87:2057–2061.

445. Minocha HC, Xue W, Reddy JR. A 50 kDa membrane protein from bovine kidney cells is a putative receptor for bovine viral diarrhea virus (BVDV). *Adv Exp Med Biol* 1997;412:145–148.

446. Miyamoto H, Okamoto H, Sato K, et al. Extraordinarily low density of hepatitis C virus estimated by sucrose density centrifugation and polymerase chain reaction. *J Gen Virol* 1992;73:715–718.

447. Mizuno M, Yamada G, Tanaka T, et al. Virion-like structures in HeLa G cells transfected with the full-length sequence of the hepatitis C virus genome. *Gastroenterology* 1995;109:1933–1940.

448. Mizushima H, Hijikata H, Asabe S-I, et al. Two hepatitis C virus glycoprotein E2 products with different C termini. *J Virol* 1994;68: 6215–6222.

449. Mizushima H, Hijikata M, Tanji Y, et al. Analysis of N-terminal processing of hepatitis C virus nonstructural protein 2. *J Virol* 1994;68: 2731–2734.

450. Moennig V, Plagemann PG. The pestiviruses. *Adv Virus Res* 1992;41: 53–98.

451. Monath TP. Japanese encephalitis—A plague of the Orient. *N Engl J Med* 1988;319:641–643.

452. Monath TP. Yellow fever: Victor, Victoria? Conqueror, conquest? Epidemics and research in the last forty years and prospects for the future. *Am J Trop Med Hyg* 1991;45:1–43.

453. Monath TP. Dengue: The risk to developed and developing countries. *Proc Natl Acad Sci U S A* 1994;91:2395–2400.

454. Monath TP, Levenbook I, Soike K, et al. Chimeric yellow fever virus 17D-Japanese encephalitis virus vaccine: Dose-response effectiveness and extended safety testing in rhesus monkeys. *J Virol* 2000;74: 1742–1751.

455. Monath TP, Soike K, Levenbook I, et al. Recombinant, chimaric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. *Vaccine* 1999;17:1869–1882.

456. Monazahian M, Bohme I, Bonk S, et al. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *J Med Virol* 1999;57:223–229.

457. Mondelli MU, Silini E. Clincal significance of hepatitis C genotypes. *J Hepatol* 1999;31:65–70.

458. Moormann RJ, Hulst MM. Hog cholera virus: Identification and characterization of the viral RNA and the virus-specific RNA synthesized in infected swine kidney cells. *Virus Res* 1988;11:281–291.

459. Moormann RJ, van Gennip HG, Miedema GK, et al. Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. *J Virol* 1996;70:763–770.

460. Moormann RJ, Warmerdam PA, van der Meer B, et al. Molecular cloning and nucleotide sequence of hog cholera virus strain Brescia and mapping of the genomic region encoding envelope protein E1. *Virology* 1990;177:184–198.

461. Moradpour D, Englert C, Wakita T, Wands JR. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* 1996;222:51–63.

462. Moradpour D, Wakita T, Wands JR, Blum HE. Tightly regulated expression of the entire hepatitis C virus structural region in continuous human cell lines. *Biochem Biophys Res Commun* 1998;246: 919–924.

463. Morgenstern KA, Landro JA, Hsiao K, et al. Polynucleotide modulation of the protease, nucleoside triphosphatase, and helicase activities of a hepatitis C virus NS3-NS4A complex isolated from transfected COS cells. *J Virol* 1997;71:3767–3775.

464. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065–1067.

465. Moriya K, Yotsuyanagi Y, Shintani Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997; 78:1527–1531.

466. Morozova OV, Tsekhanovskaya NA, Maksimova TG, et al. Phosphorylation of tick-borne encephalitis virus NS5 protein. *Virus Res* 1997; 49:9–15.

467. Moser C, Stettler P, Tratschin JD, Hofmann MA. Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. *J Virol* 1999;73:7787–7794.

468. Muerhoff AS, Leary TP, Simons JN, et al. Genomic organization of GB viruses A and B: Two new members of the *Flaviviridae* associated with GB agent hepatitis. *J Virol* 1995;69:5621–5630.

469. Muerhoff AS, Smith DB, Leary TP, et al. Identification of GB virus C variants by phylogenetic analysis of 5′-untranslated and coding region sequences [published erratum appears in *J Virol* 1997;71:8952]. *J Virol* 1997;71:6501–6508.

470. Munoz ML, Cisneros A, Cruz J, et al. Putative dengue virus receptors from mosquito cells. *FEMS Microbiol Lett* 1998;168:251–258.

471. Muramatsu S, Ishido S, Fujita T, et al. Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting the localization. *J Virol* 1997;71:4954–4961.

472. Murphy FA. Togavirus morphology and morphogenesis. In: Schlesinger RW, eds. *The Togaviruses: Biology, Structure, Replication*. New York: Academic Press, 1980:241–316.

473. Murphy FA, Harrison AK, Gary GW Jr, et al. St. Louis encephalitis virus infection in mice. Electron microscopic studies of central nervous system. *Lab Invest* 1968;19:652–662.

474. Murray JM, Aaskov JG, Wright PJ. Processing of the dengue virus type 2 proteins prM and C-prM. *J Gen Virol* 1993;74:175–182.

475. Murthy HM, Clum S, Padmanabhan R. Dengue virus NS3 serine protease. Crystal structure and insights into interaction of the active site with substrates by molecular modeling and structural analysis of mutational effects. *J Biol Chem* 1999;274:5573–5580.

476. Mussgay M, Enzmann P-J, Horzinek MC, Weiland E. Growth cycle of arboviruses in vertebrate and arthropod cells. *Prog Med Virol* 1975;19: 258–323.

477. Muylaert IR, Galler RG, Rice CM. Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: Effects on virus replication and mouse neurovirulence. *Virology* 1996;222:159–168.

478. Muylaert IR, Galler RG, Rice CM. Genetic analysis of yellow fever virus NS1 protein: Identification of a temperature-sensitive mutation which blocks RNA accumulation. *J Virol* 1997;71:291–298.

479. Nagasaka A, Hige S, Matsushima T, et al. Differential flotation centrifugation study of hepatitis C virus and response to interferon therapy. *J Med Virol* 1997;52:190–194.

480. Nathanson N. Emergence of new viral infections: Implications for the blood supply. *Biologicals* 1998;26:77–84

481. Nawa M. Effects of bafilomycin A1 on Japanese encephalitis virus in C6/36 mosquito cells. *Arch Virol* 1998;143:1555–1568.

482. Neddermann P, Clementi A, De Francesco R. Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. *J Virol* 1999;73:9984–9991

483. Nestorowicz A, Chambers TJ, Rice CM. Mutagenesis of the yellow fever virus NS2A/2B cleavage site: Effects on proteolytic processing, viral replication and evidence for alternative processing of the NS2A protein. *Virology* 1994;199:114–123.

484. Neumann AU, Lam NP, Dahari H, et al. Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;282:103–107.

485. Ng ML. Ultrastructural studies of Kunjin virus-infected *Aedes albopictus* cells. *J Gen Virol* 1987;68:577–582.

486. Ng ML, Hong SS. Flavivirus infection: Essential ultrastructural changes and association of Kunjin virus NS3 protein with microtubules. *Arch Virol* 1989;106:103–120.

487. Ng ML, Lau LC. Possible involvement of receptors in the entry of Kunjin virus into Vero cells. *Arch Virol* 1988;100:199–211.

488. Ng ML, Yeong FM, Tan SH. Cryosubstitution technique reveals new morphology of flavivirus-induced structures. *J Virol Methods* 1994; 49:305–314.

489. Nishihara T, Nozaki C, Nakatake H, et al. Secretion and purification of hepatitis C virus NS1 glycoprotein produced by recombinant baculovirus-infected insect cells. *Gene* 1993;129:207–214.

490. Nolandt O, Kern V, Muller H, et al. Analysis of hepatitis C virus core protein interaction domains. *J Gen Virol* 1997;78:1331–1340.

491. Nowak T, Färber PM, Wengler G, Wengler G. Analyses of the terminal sequences of West Nile virus structural proteins and of the *in vitro* translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. *Virology* 1989; 169:365–376.

492. Nowak T, Wengler G. Analysis of disulfides present in the membrane proteins of the West Nile flavivirus. *Virology* 1987;156:127–137.

493. Ogata N, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci U S A* 1991;88:3392–3396.

494. Oh JW, Ito T, Lai MM. A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J Virol* 1999;73:7694–7702.

495. Oh JW, Sheu GT, Lai MM. Template requirement and initiation site selection by hepatitis C virus polymerase on a minimal viral RNA template. *J Biol Chem* 2000;275:17710–17717.

496. Ohashi K, Marion PL, Nakai H, et al. Sustained survival of human hepatocytes in mice: A model for *in vivo* infection with human hepatitis B and hepatitis delta viruses. *Nat Med* 2000;6:337–341.

497. Ohyama A, Ito T, Tanimura E, et al. Electron microscopic observation of the budding maturation of group B arboviruses. *Microbiol Immunol* 1977;21:535–538.

498. Okamoto H, Kojima M, Sakamoto M, et al. The entire nucleotide sequence and classification of a hepatitis C virus isolate of a novel genotype from an Indonesian patient with chronic liver disease. *J Gen Virol* 1994;75:629–635.

499. Okamoto H, Kurai K, Okada S-I, et al. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. *Virology* 1992;188: 331–341.

500. Okamoto H, Okada S, Sugiyama Y, et al. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. *J Gen Virol* 1991,72:2697–2704.

501. Park JS, Yang JM, Min MK. Hepatitis C virus nonstructural protein NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene. *Biochem Biophys Res Commun* 2000;267:581–587.

502. Parks WP, Melnick JL. Attempted isolation of hepatitis viruses in marmosets. *J Infect Dis* 1969;120:539–547.

503. Pasquinelli C, Shoenberger JM, Chung J, et al. Hepatitis C virus core and E2 protein expression in transgenic mice. *Hepatology* 1997;25: 719–727.

504. Pasquo A, Nardi MC, Dimasi N, et al. Rational design and functional expression of a constitutively active single-chain NS4A-NS3 proteinase. *Fold Des* 1998;3:433–441.

505. Paterson M, Laxton CD, Thomas HC, et al. Hepatitis C virus NS5A protein inhibits interferon antiviral activity, but the effects do not correlate with clinical response. *Gastroenterology* 1999;117: 1187–1197.

506. Paton DJ, Lowings JP, Barrett AD. Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. *Virology* 1992;190: 763–772.

507. Pavesi A. Detection of signature sequences in overlapping genes and prediction of a novel overlapping gene in hepatitis G virus. *J Mol Evol* 2000;50:284–295.

508. Pawlotsky JM. Hepatitis C virus (HCV) NS5A protein: Role in HCV replication and resistance to interferon-alpha. *J Viral Hepat* 1999;6 (suppl 1):47–48.

509. Peiris JS, Porterfield JS. Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. *Nature* 1979;282: 509–511.

510. Peiris JS, Porterfield JS. Antibody-dependent enhancement of plaque formation on cell lines of macrophage origin—A sensitive assay for antiviral antibody. *J Gen Virol* 1981;57:119–125.

511. Peiris JS, Porterfield JS. Antibody-dependent plaque enhancement: Its antigenic specificity in relation to Togaviridae. *J Gen Virol* 1982;58: 291–296.

512. Pellerin C, Moir S, Lecomte J, Tijssen P. Comparison of the p125 coding region of bovine viral diarrhea viruses. *Vet Microbiol* 1995;45: 45–57.

513. Pellerin C, van den Hurk J, Lecomte J, Tussen P. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. *Virology* 1994;203:260–268.

514. Pestova TV, Hellen CU. Internal initiation of translation of bovine viral diarrhea virus RNA. *Virology* 1999;258:249–256.

515. Pestova TV, Shatsky IN, Fletcher SP, et al. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* 1998;12:67–83.

516. Peterson J, Dandri M, Gupta S, Rogler CE. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1998;95:310–315.

517. Pethel M, Falgout B, Lai C-J. Mutational analysis of the octapeptide sequence motif at the NS1-NS2A cleavage junction of dengue type 4 virus. *J Virol* 1992;66:7225–7231.

518. Petrie M, Yolken RH, Dubovi EJ, et al. Baculovirus expression of pestivirus non-structural proteins. *J Gen Virol* 1992;73:1867–1871.

519. Petrik J, Parker H, Alexander GJ. Human hepatic glyceraldehyde-3-phosphate dehydrogenase binds to the poly(U) tract of the 3′ non-coding region of hepatitis C virus genomic RNA. *J Gen Virol* 1999;80: 3109–3113.

520. Philipotts RJ, Stephenson JR, Porterfield JS. Antibody-dependent enhancement of tick-borne encephalitis virus infectivity. *J Gen Virol* 1985;66:1831–1837.

521. Pickering JM, Thomas HC, Karayiannis P. Genetic diversity between hepatitis G virus isolates: Analysis of nucleotide variation in the NS-3 and putative "core" peptide genes. *J Gen Virol* 1997;78:53–60.

522. Pieroni L, Santolini E, Fipaldini C, et al. *In vitro* study of the NS2-3 protease of hepatitis C virus. *J Virol* 1997;71:6373–6380.

523. Piteri P, Uematsu Y, Compagnoli S, et al. Binding of hepatitis C virus to CD81. *Science* 1998;282:938–941.

524. Pincus S, Mason PW, Konishi E, et al. Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* 1992;187:290–297.

525. Pizzi E, Tramontano A, Tomei L, et al. Molecular-model of the specificity pocket of the hepatitis C virus protease: Implications for substrate recognition. *Proc Natl Acad Sci U S A* 1994;91:888–892.

526. Pletnev AG, Men R. Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. *Proc Natl Acad Sci U S A* 1998;95:1746–1751.

527. Polo S, Ketner G, Levis R, Falgout B. Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. *J Virol* 1997;71:5366–5374.

528. Polyak SJ, Paschal DM, McArdle S, et al. Characterization of the effects of hepatitis C virus nonstructural 5A protein expression in human cell lines and on interferon-sensitive virus replication. *Hepatology* 1999;29:1262–1271.

529. Poole TL, Wang C, Popp RA, et al. Pestivirus translation initiation occurs by internal ribosome entry. *Virology* 1995;206:750–754.

530. Post PR, Carvalho R, Galler R. Glycosylation and secretion of yellow fever virus nonstructural protein NS1. *Virus Res* 1991;18:291–302.

531. Preugschat F, Averett DR, Clarke BE, Porter DJT. A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. *J Biol Chem* 1996; 271:24449–24457.

532. Preugschat F, Lenches EM, Strauss JH. Flavivirus enzyme-substrate interactions studied with chimeric proteinases: Identification of an intragenic locus important for substrate recognition. *J Virol* 1991;65: 4749–4758.

533. Preugschat F, Strauss JH. Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus *in vitro* and *in vivo*. *Virology* 1991;185: 689–697.

534. Preugschat F, Yao C-W, Strauss JH. *In vitro* processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. *J Virol* 1990;64:4364–4374.

535. Prince AM, Huima-Byron T, Parker TS, Levine DM. Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. *J Viral Hepat* 1996;3:11–17.

536. Pringle CR. Virus taxonomy—1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998 [news] [published erratum appears in *Arch Virol* 1999,144:1667]. *Arch Virol* 1999;144:421–429.

537. Proutski V, Gaunt MW, Gould EA, Holmes EC. Secondary structure of the 3′-untranslated region of yellow fever virus: Implications for virulence, attenuation and vaccine development. *J Gen Virol* 1997;78:1543–1549.

538. Proutski V, Gould EA, Holmes EC. Secondary structure of the 3′ untranslated region of flaviviruses: Similarities and differences. *Nucleic Acids Res* 1997;25:1194–1202.

539. Pryor MJ, Gualano RC, Lin B, et al. Growth restriction of dengue virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins. *J Gen Virol* 1998;79:2631–2639.

540. Pryor MJ, Wright PJ. Glycosylation mutants of dengue virus NS1 protein. *J Gen Virol* 1994;75:1183–1187.

541. Pugachev KV, Nomokonova NY, Dobrikova EY, Wolf YI. Site-directed mutagenesis of the tick-borne encephalitis virus NS3 gene reveals the putative serine protease domain of the NS3 protein. *FEBS Lett* 1993;328:115–118.

542. Purchio AF, Larson R, Collett MS. Characterization of virus-specific RNA synthesized in bovine cells infected with bovine viral diarrhea virus. *J Virol* 1983;48:320–324.

543. Purchio AF, Larson R, Torborg LL, Collett MS. Cell-free translation of bovine viral diarrhea virus RNA. *J Virol* 1984;52:973–975.

544. Qi F, Ridpath JF, Berry ES. Insertion of a bovine SMT3B gene in NS4B and duplication of NS3 in a bovine viral diarrhea virus genome correlate with the cytopathogenicity of the virus. *Virus Res* 1998;57:1–9.

545. Qi F, Ridpath JF, Lewis T, et al. Analysis of the bovine viral diarrhea virus genome for possible cellular insertions. *Virology* 1992;189:285–292.

546. Qu X, Chen W, Maguire T, Austin F. Immunoreactivity and protective effects in mice of a recombinant dengue 2 Tonga virus NS1 protein produced in a baculovirus expression system. *J Gen Virol* 1993;74:89–97.

547. Ralston R, Thudium K, Berger K, et al. Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. *J Virol* 1993;67:6753–6761.

548. Ramos-Castaneda J, Imbert JL, Barron BL, Ramos C. A 65-kDa trypsin-sensible membrane cell protein as a possible receptor for dengue virus in cultured neuroblastoma cells. *J Neurovirol* 1997;3:435–440.

549. Ramratnam B, Bonhoeffer S, Binley J, et al. Rapid production and clearance of HIV-1 and hepatitis C virus assessed by large volume plasma apheresis. *Lancet* 1999;354:1782–1785.

550. Randolph VB, Stollar V. Low pH-induced cell fusion in flavivirus-infected *Aedes albopictus* cell cultures. *J Gen Virol* 1990;71:1845–1850.

551. Randolph VB, Winkler G, Stollar V. Acidotropic amines inhibit proteolytic processing of flavivirus prM protein. *Virology* 1990;174:450–458.

552. Rauscher S, Flamm C, Mandl CW, et al. Secondary structure of the 3′-noncoding region of flavivirus genomes: Comparative analysis of base pairing probabilities. *RNA* 1997;3:779–791.

553. Ravaggi A, Natoli G, Primi D, et al. Intracellular localization of full-length and truncated hepatitis C virus core protein expressed in mammalian cells. *J Hepatol* 1994;20:833–836.

554. Ray RB, Lagging LM, Meyer K, Ray R. Hepatitis C virus core protein cooperates with *ras* and transforms primary rat embryo fibroblasts to tumorigenic phenotype. *J Virol* 1996;70:4438–4443.

555. Ray RB, Lagging LM, Meyer K, et al. Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. *Virus Res* 1995;37:209–220.

556. Ray RB, Meyer K, Ray R. Suppression of apoptotic cell death by hepatitis C virus core protein. *Virology* 1996;226:176–182.

557. Ray RB, Meyer K, Steele R, et al. Inhibition of tumor necrosis factor (TNF-α)-mediated apoptosis by hepatitis C virus core protein. *J Biol Chem* 1998;273:2256–2259.

558. Ray RB, Steele R, Meyer K, Ray R. Transcriptional repression of p53 promoter by hepatitis C virus core protein. *J Biol Chem* 1997;272:10983–10986.

559. Ray RB, Steele R, Meyer K, Ray R. Hepatitis C virus core protein represses p21/WAF1/Cip1/Sid1 promoter activity. *Gene* 1998;208:331–336.

560. Rebhun WC, French TW, Perdrizet JA, et al. Thrombocytopenia associated with acute bovine virus diarrhea infection in cattle. *J Vet Intern Med* 1989;3:42–46.

561. Reed KE, Gorbalenya AE, Rice CM. The NS5A/NS5 proteins of viruses from three genera of the family *Flaviviridae* are phosphorylated by associated serine/threonine kinases. *J Virol* 1998;72:6199–6206.

562. Reed KE, Grakoui A, Rice CM. The hepatitis C virus NS2-3 autoproteinase: Cleavage site mutagenesis and requirements for bimolecular cleavage. *J Virol* 1995;69:4127–4136.

563. Reed KE, Rice CM. Identification of the major phosphorylation site of the hepatitis C virus H strain NS5A protein as serine 2321. *J Biol Chem* 1999;274:28011–28018.

564. Reed KE, Xu J, Rice CM. Phosphorylation of the hepatitis C virus NS5A protein *in vitro* and *in vivo*: Properties of the NS5A-associated kinase. *J Virol* 1997;71:7187–7197.

565. Reed W, Carroll J. The etiology of yellow fever. A supplemental note. *Am Med* 1902;3:301–305.

566. Reed W, Carroll J, Agramonte A. The etiology of yellow fever. An additional note. *JAMA* 1901;36:431–440.

567. Rey F, Heinz FX, Mandl C, et al. Crystal structure of the envelope glycoprotein E from tick borne encephalitis virus. *Nature* 1995;375:291–298.

568. Reynolds JE, Kaminski A, Carroll AR, et al. Internal initiation of translation of hepatitis C virus RNA: The ribosome entry site is at the authentic initiation codon. *RNA* 1996;2:867–878.

569. Reynolds JE, Kaminski A, Kettinen HJ, et al. Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO J* 1995;14:6010–6020.

570. Rice CM. Is CD81 the key to hepatitis C virus entry? *Hepatology* 1999;29:990–992.

571. Rice CM, Aebersold R, Teplow DB, et al. Partial N-terminal amino acid sequences of three nonstructural proteins of two flaviviruses. *Virology* 1986;151:1–9.

572. Rice CM, Grakoui A, Galler R, Chambers TJ. Transcription of infectious yellow fever virus RNA from full-length cDNA templates produced by *in vitro* ligation. *New Biol* 1989;1:285–296.

573. Rice CM, Lenches EM, Eddy SR, et al. Nucleotide sequence of yellow fever virus: Implications for flavivirus gene expression and evolution. *Science* 1985;229:726–733.

574. Ridpath JF, Bolin SR. The genomic sequence of a virulent bovine viral diarrhea virus (BVDV) from the type 2 genotype: Detection of a large genomic insertion in a noncytopathic BVDV. *Virology* 1995;212:39–46.

575. Ridpath JF, Bolin SR. Comparison of the complete genomic sequence of the border disease virus, BD31, to other pestiviruses. *Virus Res* 1997;50:237–243.

576. Ridpath JF, Bolin SR, Dubovi EJ. Segregation of bovine viral diarrhea virus into genotypes. *Virology* 1994;205:66–74.

577. Rijnbrand R, Abell G, Lemon SM. Mutational analysis of the GB virus B internal ribosome entry site. *J Virol* 2000;74:773–783.

578. Rijnbrand R, Bredenbeek PJ, van der Straaten T, et al. Almost the entire 5′ non-translated region of hepatitis C virus is required for cap-independent translation. *FEBS Lett* 1995;365:115–119.

579. Rijnbrand R, van der Straaten T, van Rijn PA, et al. Internal entry of ribosomes is directed by the 5′ noncoding region of classical swine fever virus and is dependent on the presence of an RNA pseudoknot upstream of the initiation codon. *J Virol* 1997;71:451–457.

580. Rijnbrand RC, Abbink TE, Haasnoot PC, et al. The influence of AUG codons in the hepatitis C virus 5′ nontranslated region on translation initiation and mapping of the translation initiation window. *Virology* 1996;226:47–56.

581. Rijnbrand RCA, Lemon SM. Internal ribosome entry site–mediated translation in hepatitis C virus replication. In: Hagedorn C, Rice CM, eds. *Hepatitis C Virus*. Berlin: Springer-Verlag, 2000:85–116.

582. Robertson B, Myers G, Howard C, et al. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: Proposals for standardization. International Committee on Virus Taxonomy [news]. *Arch Virol* 1998;143:2493–503.

583. Roehrig JT, Johnson AJ, Hunt AR, et al. Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation. *Virology* 1990;177:668–675.

584. Rothman AL, Ennis FA. Immunopathogenesis of Dengue hemorrhagic fever. *Virology* 1999;257:1–6.

585. Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. *Virology* 1997;229:68–76.

586. Ruggli N, Tratschin JD, Mittelholzer C, Hofmann MA. Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J Virol* 1996;70:3478–3487.

587. Rümenapf T, Meyers G, Stark R, Thiel H-J. Hog cholera virus—Characterization of specific antiserum and identification of cDNA clones. *Virology* 1989;171:18–27.

588. Rümenapf T, Meyers G, Stark R, Thiel H-J. Molecular characterization of hog cholera virus. *Arch Virol Suppl* 1991;3:7–18.
589. Rümenapf T, Stark R, Heimann M, Thiel H-J. N-terminal protease of pestiviruses: Identification of putative catalytic residues by site-directed mutagenesis. *J Virol* 1998;72:2544–2547.
590. Rümenapf T, Stark R, Meyers G, Thiel H-J. Structural proteins of hog cholera virus expressed by vaccinia virus: Further characterization and induction of protective immunity. *J Virol* 1991;65:589–597.
591. Rümenapf T, Unger G, Strauss JH, Thiel H-J. Processing of the envelope glycoproteins of pestiviruses. *J Virol* 1993;67:3288–3294.
592. Russell PK, Brandt WE, Dalrymple JM. Chemical and antigenic structure of flaviviruses. In: Schlesinger RW, eds. *The Togaviruses: Biology, Structure, Replication.* New York: Academic Press, 1980: 503–529.
593. Ryu W-S, Choi D-Y, Yang J-Y, et al. Characterization of the putative E2 envelope glycoprotein of hepatitis C virus expressed in stably transformed Chinese hamster ovary cells. *Mol Cells* 1995;5:563–568.
594. Saito I, Miyamura T, Ohbayashi A, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990;87:6547–6549.
595. Sakamuro D, Furukawa T, Takegami T. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. *J Virol* 1995;69:3893–3896.
596. Salas-Benito JS, del Angel RM. Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. *J Virol* 1997;71: 7246–7252.
597. Sangar DV, Carroll AR. A tale of two strands: Reverse-transcriptase polymerase chain reaction detection of hepatitis C virus replication. *Hepatology* 1998;28:1173–1176.
598. Sangster MY, Heliams DB, MacKenzie JS, Shellam GR. Genetic studies of flavivirus resistance in inbred strains derived from wild mice: Evidence for a new resistance allele at the flavivirus resistance locus (Flv). *J Virol* 1993;67:340–347.
599. Sangster MY, Mackenzie JS, Shellam GR. Genetically determined resistance to flavivirus infection in wild *Mus musculus domesticus* and other taxonomic groups in the genus *Mus*. *Arch Virol* 1998;143: 697–715.
600. Sangster MY, Shellam GR. Genetically controlled resistance to flaviviruses within the house mouse complex of species. *Curr Top Microbiol Immunol* 1986;127:313–318.
601. Sangster MY, Urosevic N, Mansfield JP, et al. Mapping the Flv locus controlling resistance to flaviviruses on mouse chromosome 5. *J Virol* 1994;68:448–452.
602. Santolini E, Migliaccio G, La Monica N. Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J Virol* 1994;68: 3631–3641.
603. Santolini E, Pacini L, Fipaldini C, et al. The NS2 protein of hepatitis C virus is a transmembrane polypeptide. *J Virol* 1995;69:7461–7471.
604. Sato K, Okamoto H, Aihara S, et al. Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. *Virology* 1993;196:354–357.
605. Sato K, Tanaka T, Okamoto H, et al. Association of circulating hepatitis G virus with lipoproteins for a lack of binding with antibodies. *Biochem Biophys Res Commun* 1996;229:719–725.
606. Satoh S, Hirota M, Noguchi T, et al. Cleavage of hepatitis C virus nonstructural protein 5A by a caspase-like protease(s) in mammalian cells. *Virology* 2000;270:476–487.
607. Satoh S, Tanji Y, Hijikata M, et al. The N-terminal region of hepatitis C virus nousstructural protein 3 (NS3) is essential for stable complex formation with NS4A. *J Virol* 1995;69:4255–4260.
608. Sawyer WA, Lloyd W. The use of mice in tests of immunity against yellow fever. *J Exp Med* 1931;54:533–555.
609. Sbardellati A, Scarselli E, Tomei L, et al. Identification of a novel sequence at the 3′ end of the GB virus B genome. *J Virol* 1999;73: 10546–10550.
610. Scarselli E, Urbani A, Sbardellati A, et al. GB virus B and hepatitis C virus NS3 serine proteases share substrate specificity. *J Virol* 1997;71: 4985–4989.
611. Schalich J, Allison SL, Stiasny K, et al. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. *J Virol* 1996;70:4549–4557.
612. Schein CH. From housekeeper to microsurgeon: The diagnostic and therapeutic potential of ribonucleases [published erratum appears in *Nat Biotechnol* 1997;15:927]. *Nat Biotechnol* 1997;15:529–536.
613. Schelp C, Greiser-Wilke I, Wolf G, et al. Identification of cell membrane proteins linked to susceptibility to bovine viral diarrhoea virus infection. *Arch Virol* 1995;140:1997–2009.
614. Schlauder GG, Dawson GJ, Simons JN, et al. Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J Med Virol* 1995;46:81–90.
615. Schlauder GG, Pilot-Matias TJ, Gabriel GS, et al. Origin of GB-hepatitis viruses [letter, comment]. *Lancet* 1995;346:447–448.
616. Schlesinger JJ, Brandriss MW. 17D yellow fever virus infection of P388D1 cells mediated by monoclonal antibodies: Properties of the macrophage Fc receptor. *J Gen Virol* 1983;64:1255–1262.
617. Schlesinger JJ, Brandriss MW, Cropp CB, Monath TP. Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. *J Virol* 1986;60:1153–1155.
618. Schlesinger JJ, Brandriss MW, Walsh EE. Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. *J Immunol* 1985;135:2805–2809.
619. Schlesinger JJ, Foltzer M, Chapman S. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. *Virology* 1993;192:132–141.
620. Schneider R, Unger G, Stark R, et al. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science* 1993;261: 1169–1171.
621. Schweizer M, Peterhans E. Oxidative stress in cells infected with bovine viral diarrhoea virus: A crucial step in the induction of apoptosis. *J Gen Virol* 1999;80:1147–1155.
622. Sekiya H, Kato N, Ootsuyama Y, et al. Genetic alterations of the putative envelope proteins encoding region of the hepatitis C virus in the progression to relapsed phase from acute hepatitis: Humoral immune response to hypervariable region 1. *Int J Cancer* 1994;57: 664–670.
623. Selby MJ, Glazer E, Masiarz F, Houghton M. Complex processing and protein:protein interactions in the E2:NS2 region of HCV. *Virology* 1994;204:114–122.
624. Semprini AE, Persico T, Thiers V, et al. Absence of hepatitis C virus and detection of hepatitis G virus/GB virus C RNA sequences in the semen of infected men. *J Infect Dis* 1998;177:848–854.
625. Serafino A, Valli MB, Alessandrini A, et al. Ultrastructural observations of viral particles within hepatitis C virus-infected human B lymphoblastoid cell line. *Res Virol* 1997;148:133–139.
626. Shapiro D, Brandt WE, Russell PK. Change involving a viral membrane glycoprotein during morphogenesis of group B arboviruses. *Virology* 1972;50:906–911.
627. Shi PY, Li W, Brinton MA. Cell proteins bind specifically to West Nile virus minus-strand 3′ stem-loop RNA. *J Virol* 1996;70:6278–6287.
628. Shih C-M, Chen C-M, Chen S-Y, Lee Y-HW. Modulation of the transsuppression activity of hepatitis C virus core protein by phosphorylation. *J Virol* 1995;69:1160–1171.
629. Shih CM, Lo SJ, Miyamura T, et al. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. *J Virol* 1993;67:5823–5832.
630. Shimizu Y, Yamaji K, Masuho Y, et al. Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. *J Virol* 1996;70:127–132.
631. Shimizu Y, Yoshikura H. Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons. *J Virol* 1994; 68:8406–8408.
632. Shimizu YK, Feinstone SM, Kohara M, et al. Hepatitis C virus: Detection of intracellular virus particles by electron microscopy. *Hepatology* 1996;23:205–209.
633. Shimizu YK, Igarashi H, Kiyohara T, et al. A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology* 1996;223:409–412.
634. Shimoike T, Mimori S, Tani H, et al. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J Virol* 1999;73:9718–9725.
635. Shimotohno K. Hepatocellular carcinoma in Japan and its linkage to infection with hepatitis C virus. *Semin Virol* 1993;4:305–312.
636. Simmonds P. Variability of hepatitis C virus genome. *Curr Stud Hematol Blood Transfus* 1994;61:12–35.
637. Simmonds P, Smith DB. Structural constraints on RNA virus evolution. *J Virol* 1999;73:5787–5794.

638. Simons JN, Desai SM, Mushahwar IK. The GB viruses. *Curr Top Microbiol Immunol* 2000;242:341–375.
639. Simons JN, Desai SM, Schultz DE, et al. Translation initiation in GB viruses A and C: Evidence for internal ribosome entry and implications for genome organization. *J Virol* 1996;70:6126–6135.
640. Simons JN, Leary TP, Dawson GJ, et al. Isolation of novel virus-like sequences associated with human hepatitis. *Nat Med* 1995;1:564–569.
641. Simons JN, Pilcmatas TJ, Leary TP, et al. Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc Natl Acad Sci U S A* 1995;92:3401–3405.
642. Sizova DV, Kolupaeva VG, Pestova TV, et al. Specific interaction of eukaryotic translation initiation factor 3 with the 5′ nontranslated regions of hepatitis C virus and classical swine fever virus RNAs. *J Virol* 1998;72:4775–4782.
643. Smith DB, Basaras M, Frost S, et al. Phylogenetic analysis of GBV-C/hepatitis G virus. *J Gen Virol* 2000;81:769–780.
644. Smith DB, Cuceanu N, Davidson F, et al. Discrimination of hepatitis G virus/GBV-C geographical variants by analysis of the 5′ non-coding region. *J Gen Virol* 1997;78:1533–1542.
645. Smith DB, Mellor J, Jarvis LM, and Group THCS. Variation of the hepatitis C virus 5′ non-coding region: Implications for secondary structure, virus detection and typing. *J Gen Virol* 1995;76:1749–1761.
646. Smith GW, Wright PJ. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and *Aedes albopictus* cells. *J Gen Virol* 1985;66:559–571.
647. Song J, Fujii M, Wang F, et al. The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon. *J Gen Virol* 1999; 80:879–886.
648. Speaie RR, Alexander D, Rugroden ME, et al. Characterization of the hepatitis E2/NS1 gene product expressed in mammalian cells. *Virology* 1992;188:819–830.
649. Srinivas RV, Ray RB, Meyer K, Ray R. Hepatitis C virus core protein inhibits human immunodeficiency virus type 1 replication. *Virus Res* 1996;45:87–92.
650. Sriurairatna S, Bhamarapravati N. Replication of dengue-2 virus in *Aedes albopictus* mosquitoes. *Am J Trop Med Hyg* 1977;26:1199–1205.
651. Sriurairatna S, Bhamarapravati N, Phalavadhtana O. Dengue virus infection of mice: Morphology and morphogenesis of dengue type 2 virus in suckling mouse neurones. *Infect Immun* 1973;8:1017–1028.
652. Srivastava SP, Kumar KU, Kaufman RJ. Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *J Biol Chem* 1998;273:2416–2423.
653. Stadler K, Allison SL, Schalich J, Heinz FX. Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol* 1997;71:8475–8481.
654. Stark K, Bienzle U, Hess G, et al. Detection of the hepatitis G virus genome among injecting drug users, homosexual and bisexual men, and blood donors. *J Infect Dis* 1996;174:1320–1323.
655. Stark R, Meyers G, Rümenapf T, Thiel H-J. Processing of pestivirus polyprotein: Cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. *J Virol* 1993;67:7088–7095.
656. Stark R, Rümenapf T, Meyers G, Thiel H-J. Genomic localization of hog cholera virus glycoproteins. *Virology* 1990;174:286–289.
657. Steffens S, Thiel HJ, Behrens SE. The RNA-dependent RNA polymerases of different members of the family *Flaviviridae* exhibit similar properties *in vitro*. *J Gen Virol* 1999;80:2583–2590.
658. Stempniak M, Hostomska Z, Nodes BR, Hostomsky Z. The NS3 proteinase domain of hepatitis C virus is a zinc-containing enzyme. *J Virol* 1997;71:2881–2886.
659. Stiasny K, Allison SL, Marchler-Bauer A, et al. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. *J Virol* 1996;70:8142–8147.
660. Stocks CE, Lobigs M. Posttranslational signal peptidase cleavage at the flavivirus C-prM junction *in vitro*. *J Virol* 1995;69:8123–8126.
661. Stocks CE, Lobigs M. Signal peptidase cleavage at the flavivirus C-prM junction: Dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. *J Virol* 1998;72:2141–2149.
662. Stohlman SA, Wisseman CL Jr, Eylar OR, Silverman DJ. Dengue virus-induced modifications of host cell membranes. *J Virol* 1975;16: 1017–1026.
663. Stollar V. Togaviruses in cultured arthropod cells. In: Schlesinger RW, ed. *The Togaviruses—Biology, Structure, Replication*. New Yorlc Academic Press, 1980:584–621.
664. Strode GK. *Yellow fever*. New York: McGraw-Hill, 1951.
665. Sumiyoshi H, Hoke CH, Trent DW. Infectious Japanese encephalitis virus RNA can be synthesized from *in vitro*–ligated cDNA templates. *J Virol* 1992;66:5425–5431.
666. Sumiyoshi H, Mori C, Fuke I, et al. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* 1987;161: 497–510.
667. Summers PL, Cohen WH, Ruiz MM, et al. Flaviviruses can mediate fusion from without in *Aedes albopictus* mosquito cell cultures. *Virus Res* 1989;12:383–392.
668. Susa M, Konig M, Saalmuller A, et al. Pathogenesis of classical swine fever: B-lymphocyte deficiency caused by hog cholera virus. *J Virol* 1992;66:1171–1175.
669. Suzich JA, Tamura JK, Palmer-Hill F, et al. Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *J Virol* 1993; 67:6152–6158.
670. Suzuki R, Matsuura Y, Suzuki T, et al. Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. *J Gen Virol* 1995;76:53–61.
671. Tabor E, Garety RJ, Drucker JA, et al. Transmission of non-A, non-B hepatitis from man to chimpanzee. *Lancet* 1978;1:463–466.
672. Tacke M, Kiyosawa K, Stark K, et al. Detection of antibodies to a putative hepatitis G virus envelope protein [see comments] [published erratum appears in *Lancet* 1997;349:736]. *Lancet* 1997;349:318–320.
673. Tacke M, Schmolke S, Schlueter V, et al. Humoral immune response to the E2 protein of hepatitis G virus is associated with long-term recovery from infection and reveals a high frequency of hepatitis G virus exposure among healthy blood donors. *Hepatology* 1997;26: 1626–1633.
674. Tai C-L, Chi W-K, Chen D-S, Hwang L-H. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J Virol* 1996;70:8477–8484.
675. Takahashi K, Kishimoto S, Yoshizawa H, et al. p26 protein and 33-nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected hosts. *Virology* 1992;191:431–434.
676. Takamizawa A, Mori C, Fuke I, et al. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J Virol* 1991; 65:1105–1113.
677. Takegami T, Washizu M, Yasui K. Nucleotide sequence at the 3′ end of Japanese encephalitis virus genome RNA. *Virology* 1986;152: 483–486.
678. Takikawa S, Ishii K, Aizaki H, et al. Cell fusion activity of hepatitis C virus envelope proteins. *J Virol* 2000;74:5066–5074.
679. Tameda Y, Kosaka Y, Tagawa S, et al. Infection with GB virus C (GBV-C) in patients with fulminant hepatitis. *J Hepatol* 1996;25: 842–847.
680. Tamura JK, Warrener P, Collett MS. RNA-stimulated NTPase activity associated with the p80 protein of the pestivirus bovine viral diarrhea virus. *Virology* 1993;193:1–10.
681. Tan BH, Fu J, Sugrue RJ, et al. Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. *Virology* 1996;216:317–325.
682. Tan S-L, Nakao H, Ide Y, et al. NS5A, a non-structural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling. *Proc Natl Acad Sci U S A* 1999;96: 5533–5538.
683. Tanaka T, Kato N, Cho M-J, Shimotohno K. A novel sequence found at the 3′ terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* 1995;215:744–749.
684. Tanaka T, Kato N, Cho M-J, et al. Structure of the 3′ terminus of the hepatitis C virus genome. *J Virol* 1996;70:3307–3312.
685. Tanaka T, Kato N, Nakagawa M, et al. Molecular cloning of hepatitis C virus genome from a single Japanese carrier: Sequence variation within the same individual and among infected individuals. *Virus Res* 1992;23:39–53.
686. Tanaka Y, Shimoike T, Ishii K, et al. Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5′ untranslated region of the viral genome. *Virology* 2000;270:229–236.
687. Tanimoto A, Ide Y, Arima N, et al. The amino terminal deletion mutants of hepatitis C virus nonstructural protein NS5A function as transcriptional activators in yeast. *Biochem Biophys Res Commun* 1997;236:360–364.

688. Tanji Y, Hijikata M, Hirowatari Y, Shimotohno K. Hepatitis C virus polyprotein processing: Kinetics and mutagenic analysis of serine proteinase-dependent cleavage. *J Virol* 1994;68:8418–8422.

689. Tanji Y, Hijikata M, Hirowatari Y, Shimotohno K. Identification of the domain required for trans-cleavage activity of hepatitis C viral serine proteinase. *Gene* 1994;145:215–219.

690. Tanji Y, Hijikata M, Satoh S, et al. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. *J Virol* 1995;69:1575–1581.

691. Tanji Y, Kaneko T, Satoh S, Shimotohno K. Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. *J Virol* 1995;69: 3980–3986.

692. Taremi SS, Beyer B, Maher M, et al. Construction, expression, and characterization of a novel fully activated recombinant single-chain hepatitis C virus protease. *Protein Sci* 1998;7:2143–2149.

693. Tautz N, Elbers K, Stoll D, et al. Serine protease of pestiviruses: Determination of cleavage sites. *J Virol* 1997;71:5415–5422.

694. Tautz N, Harada T, Kaiser A, et al. Establishment and characterization of cytopathogenic and noncytopathogenic pestivirus replicons. *J Virol* 1999;73:9422–9432.

695. Tautz N, Kaiser A, Thiel HJ. NS3 serine protease of bovine viral diarrhea virus: Characterization of active site residues, NS4A cofactor domain, and protease-cofactor interactions. *Virology* 2000;273:351–363.

696. Tautz N, Meyers G, Stark R, et al. Cytopathogenicity of a pestivirus correlates with a 27-nucleotide insertion. *J Virol* 1996;70:7851–7858.

697. Tautz N, Meyers G, Thiel H-J. Processing of poly-ubiquitin in the polyprotein of an RNA virus. *Virology* 1993;197:74–85.

698. Tautz N, Thiel H-J, Dubovi EJ, Meyers G. Pathogenesis of mucosal disease: A cytopathogenic pestivirus generated by an internal deletion. *J Virol* 1994;68:3289–3297.

699. Taylor DR, Shi ST, Romano PR, et al. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein [see comments]. *Science* 1999;285:107–110.

700. Teo KF, Wright PJ. Internal proteolysis of the NS3 protein specified by dengue virus 2. *J Gen Virol* 1997;78:337–341.

701. Theiler M, Smith HH. Use of yellow fever virus modified by *in vitro* cultivation for human immunization. *J Exp Med* 1937;65:787–800.

702. Thiel H-J, Plagemann PGW, Moennig V. Pestiviruses. In: Fields BN, Knipe DM, Howley PM, eds. *Fields Virology*. New York: Raven Press, 1996:1059–1073.

703. Thiel H-J, Stark R, Weiland E, et al. Hog cholera virus: Molecular composition of virions from a pestivirus. *J Virol* 1991;65:4705–4712.

704. Thomssen R, Bonk S, Propfe C, et al. Association of hepatitis C virus in human sera with beta-lipoprotein. *Med Microbiol Immunol* 1992; 181:293–300.

705. Thomssen R, Bonk S, Thiele A. Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. *Med Microbiol Immunol* 1993;182:329–334.

706. Timofeev AV, Ozherelkov SV, Pronin AV, et al. Immunological basis for protection in a murine model of tick-borne encephalitis by a recombinant adenovirus carrying the gene encoding the NS1 nonstructural protein. *J Gen Virol* 1998;79:689–695.

707. Tomei L, Failla C, Santolini E, et al. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol* 1993;67: 4017–4026.

708. Tomei L, Failla C, Vitale RL, et al. A central hydrophobic domain of the hepatitis C virus NS4A protein is necessary and sufficient for the activation of the NS3 protease. *J Gen Virol* 1996;77:1065–1070.

709. Tomori O. Impact of yellow fever on the developing world. *Adv Virus Res* 1999;53:5–34.

710. Tratschin JD, Moser C, Ruggli N, Hofmann MA. Classical swine fever virus leader proteinase N^{pro} is not required for viral replication in cell culture. *J Virol* 1998;72:7681–7684.

711. Trent DW. Antigenic characterization of flavivirus structural proteins separated by isoelectric focusing. *J Virol* 1977;22:608–618.

712. Trent DW, Naeve CW. Biochemistry and replication. In: Monath T, ed. *St. Louis Encephalitis*. Washington, DC: American Public Health Association, 1980:159–199.

713. Trent DW, Swensen CC, Qureshi AA. Synthesis of Saint Louis encephalitis virus ribonucleic acid in BHK-21-13 cells. *J Virol* 1969; 3:385–394.

714. Tsuchihara K, Hijikata M, Fukuda K, et al. Hepatitis C virus core protein regulates cell growth and signal transduction pathway transmitting growth stimuli. *Virology* 1999;258:100–107.

715. Tsuchihara K, Tanaka T, Hijikata M, et al. Specific interaction of polypyrimidine tract-binding protein with the extreme 3′-terminal structure of the hepatitis C virus genome, the 3′X. *J Virol* 1997;71: 6720–6726.

716. Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A. Internal ribosome entry site within hepatitis C virus RNA. *J Virol* 1992;66: 1476–1483.

717. Tu H, Gao L, Shi ST, et al. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 1999;263:30–41.

718. Tucker TJ, Smuts HE, Knobel GD, et al. Evidence that GBV-C/hepatitis G virus is primarily at lymphotropic virus. *J Viral Hepat* 2000;61: 52–58.

719. Urosevic N, Mansfield JP, Mackenzie JS, Shellam GR. Low resolution mapping around the flavivirus resistance locus (Flv) on mouse chromosome 5. *Mamm Genome* 1995;6:454–458.

720. Urosevic N, Silvia OJ, Sangster MY, et al. Development and characterization of new flavivirus-resistant mouse strains bearing Flv(r)-like and Flv(rm) alleles from wild or wild-derived mice. *J Gen Virol* 1999;80:897–906.

721. Urosevic N, van Maanen M, Mansfield JP, et al. Molecular characterization of virus-specific RNA produced in the brains of flavivirus-susceptible and -resistant mice after challenge with Murray Valley encephalitis virus. *J Gen Virol* 1997;78:23–29.

722. Valle RP, Falgout B. Mutagenesis of the NS3 protease of dengue virus type 2. *J Virol* 1998;72:624–632.

723. van Rijn PA, van Gennip HG, de Meijer EJ, Moormann RJ. Epitope mapping of envelope glycoprotein E1 of hog cholera virus strain Brescia. *J Gen Virol* 1993;74:2053–2060.

724. van Rijn PA, van Gennip HG, Leendertse CH, et al. Subdivision of the pestivirus genus based on envelope glycoprotein E2. *Virology* 1997; 237:337–348.

725. van Zijl M, Wensvoort G, de Kluyver E, et al. Live attenuated pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus protects swine against both pseudorabies and hog cholera. *J Virol* 1991;65:2761–2765.

726. Varnavski AN, Khromykh AA. Noncytopathic flavivirus replicon RNA-based system for expression and delivery of heterologous genes. *Virology* 1999;255:366–375.

727. Vassilev VB, Collett MS, Donis RO. Authentic and chimeric full-length genomic cDNA clones of bovine viral diarrhea virus that yield infectious transcripts. *J Virol* 1997,71:471–478.

728. Vazzov S, Riffelmann M, Khoudyakov Y, et al. Genetic heterogeneity of hepatitis G virus isolates from different parts of the world. *J Gen Virol* 1997;78:577–581.

729. Wahlberg JM, Boere WAM, Garoff H. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J Virol* 1989;63: 4991–4997.

730. Wahlberg JM, Bron R, Wilschut J, Garoff H. Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. *J Virol* 1992;66:7309–7318.

731. Wahlberg JM, Garoff H. Membrane fusion process of Semliki Forest virus I: Low pH–induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. *J Cell Biol* 1992;116:339–348.

732. Wakita T, Katsume A, Kato J, et al. Possible role of cytotoxic T cells in acute liver injury in hepatitis C virus cDNA transgenic mice mediated by Cre/loxP system. *J Med Virol* 2000;62:308–317.

733. Wakita T, Taya C, Katsume A, et al. Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. *J Biol Chem* 1998;273:9001–9006.

734. Walker CM. Comparative features of hepatitis C virus infection in humans and chimpanzees. *Springer Semin Immunopathol* 1997;19: 85–98.

735. Wallner G, Mandl CW, Kunz C, Heinz FX. The flavivirus 3′-noncoding region: Extensive size heterogeneity independent of evolutionary relationships among strains of tick-borne encephalitis virus. *Virology* 1995;213:169–178.

736. Wang C, Le SY, Ali N, Siddiqui A. An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5′ noncoding region. *RNA* 1995;1:526–537.

737. Wang C, Sarnow P, Siddiqui A. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. *J Virol* 1993;67:3338–3344.

738. Wang C, Siddiqui A. Structure and function of the hepatitis C virus internal ribosome entry site. *Curr Top Microbiol Immunol* 1995;203: 99–115.
739. Wang E, Weaver SC, Shope RE, et al. Genetic variation in yellow fever virus: Duplication in the 3′ noncoding region of strains from Africa. *Virology* 1996;225:274–281.
740. Wang JJ, Liao CL, Yang CI, et al. Localizations of NS3 and E proteins in mouse brain infected with mutant strain of Japanese encephalitis virus. *Arch Virol* 1998;143:2353–2369.
741. Wang S, He R, Anderson R. PrM- and cell-binding domains of the dengue virus E protein. *J Virol* 1999;73:2547–2551.
742. Wang Y, Okamoto H, Tsuda F, et al. Prevalence, genotypes, and an isolate (HC-C2) of hepatitis C virus in Chinese patients with liver disease. *J Med Virol* 1993;40:254–260.
743. Warrener P, Collett MS. Pestivirus NS3 (p80) protein possesses RNA helicase activity. *J Virol* 1995;69:1720–1726.
744. Warrener P, Tamura JK, Collett MS. An RNA-stimulated NTPase activity associated with yellow fever virus NS3 protein expressed in bacteria. *J Virol* 1993;67:989–996.
745. Watson JP, Bevitt DJ, Spickett GP, et al. Hepatitis C virus density heterogeneity and viral titre in acute and chronic infection: A comparison of immunodeficient and immunocompetent patients. *J Hepatol* 1996;25:599–607.
746. Weiland E, Ahl R, Stark R, et al. A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. *J Virol* 1992;66:3677–3682.
747. Weiland E, Stark R, Haas B, et al. Pestivirus glycoprotein which induces neutralizing antibodies form part of a disulfide-linked heterodimer. *J Virol* 1990;64:3563–3569.
748. Weiland F, Weiland E, Unger G, et al. Localization of pestiviral envelope proteins E(rns) and E2 at the cell surface and on isolated particles. *J Gen Virol* 1999;80:1157–1165.
749. Weiner AJ, Brauer MJ, Rosenblatt J, et al. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology* 1991;180:842–848.
750. Weiner AJ, Geysen HM, Christopherson C, et al. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections. *Proc Natl Acad Sci U S A* 1992;89:3468–3472.
751. Wengler G, Castle E. Analysis of structural properties which possibly are characteristics for the 3′-terminal sequences of the genome RNA of flaviviruses. *J Gen Virol* 1986;67:1183–1188.
752. Wengler G, Czaya G, Färber PM, Hegemann JH. *In vitro* synthesis of West Nile virus proteins indicates that the amino-terminal segment of the NS3 protein contains the active centre of the protease which cleaves the viral polyprotein after multiple basic amino acids. *J Gen Virol* 1991;72:851–858.
753. Wengler G, Wengler G. Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J Virol* 1989;63: 2521–2526.
754. Wengler G, Wengler G. The carboxy-terminal part of the NS3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. *Virology* 1991;184:707–715.
755. Wengler G, Wengler G. The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* 1993;197:265–273.
756. Wengler G, Wengler G, Gross HJ. Studies on virus-specific nucleic acids synthesized in vertebrate and mosquito cells infected with flaviviruses. *Virology* 1978;89:423–437.
757. Wengler G, Wengler G, Nowak T, Castle E. Description of a procedure which allows isolation of viral nonstructural proteins from BHK vertebrate cells infected with the West Nile flavivirus in a state which allows their direct chemical characterization. *Virology* 1990;177:795–801.
758. Wensvoort G. Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. *J Gen Virol* 1989;70: 2865–2876.
759. Westaway EG. Replication of flaviviruses. In: Schlesinger RW, ed. *The Togaviruses: Biology, Structure, Replication.* New York: Academic Press, 1980:531–581.
760. Westaway EG. Flavivirus replication strategy. *Adv Virus Res* 1987;33: 45–90.
761. Westaway EG, Brinton MA, Gaidamovich SY, et al. *Flaviviridae. Intervirology* 1985;24:183–192.
762. Westaway EG, Khromykh AA, Kenney MT, et al. Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. *Virology* 1997;234:31–41.
763. Westaway EG, Khromykh AA, Mackenzie JM. Nascent flavivirus RNA colocalized in situ with double-stranded RNA in stable replication complexes. *Virology* 1999;258:108–117.
764. Westaway EG, Mackenzie JM, Kenney MT, et al. Ultrastructure of Kunjin virus-infected cells: Colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *J Virol* 1997;71:6650–6661.
765. Whitfield SG, Murphy FA, Sudia WD. St. Louis encephalitis virus: An ultrastructural study of infection in a mosquito vector. *Virology* 1973;56:70–87.
766. Widjojoatmodjo MN, van Gennip HG, Bouma A, et al. Classical swine fever virus E(rns) deletion mutants: Trans-complementation and potential use as nontransmissible, modified, live-attenuated marker vaccines. *J Virol* 2000;74:2973–2980.
767. Windisch JM, Schneider R, Stark R, et al. RNase of classical swine fever virus: Biochemical characterization and inhibition by virus-neutralizing monoclonal antibodies. *J Virol* 1996;70:352–358.
768. Winkler G, Heinz FX, Kunz C. Studies on the glycosylation of flavivirus E proteins and the role of carbohydrate in antigenic structure. *Virology* 1987;159:237–243.
769. Winkler G, Maxwell SE, Ruemmler C, Stollar V. Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. *Virology* 1989;171:302–305.
770. Winkler G, Randolph VB, Cleaves GR, et al. Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology* 1988;162:187–196.
771. Wiskerchen M, Belzer SK, Collett MS. Pestivirus gene expression: The first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity. *J Virol* 1991;65: 4508–4514.
772. Wiskerchen M, Collett MS. Pestivirus gene expression: Protein p80 of bovine viral diarrhea virus is a proteinase involved in polyprotein processing. *Virology* 1991;184:341–350.
773. Wölk B, Sansonno D, Kräusslich H-G, et al. Subcellular localization, stability and trans-cleavage of hepatitis C virus NS3-4A complex expressed in tetracyclin-regulated cell lines. *J Virol* 2000;74: 2293–2304.
774. Worobey M, Rambaut A, Holmes EC. Widespread intra-serotype recombination in natural populations of dengue virus. *Proc Natl Acad Sci U S A* 1999;96:7352–7357.
775. Wu RR, Mizokami M, Cao K, et al. GB virus C/hepatitis G virus infection in southern China. *J Infect Dis* 1997;175:168–171.
776. Xiang J, Daniels KJ, Soll DR, et al. Visualization and characterization of GB-virus-C particles: Evidence for a nucleocapsid. *J Viral Hepat* 1999;6(Suppl 1):16–22.
777. Xiang J, Klinzman D, McLinden J, et al. Characterization of hepatitis G virus (GB-C virus) particles: Evidence for a nucleocapsid and expression of sequences upstream of the E1 protein. *J Virol* 1998;72: 2738–2744.
778. Xie ZC, Riezu-Boj JI, Lasarte JJ, et al. Transmission of hepatitis C virus infection to tree shrews. *Virology* 1998;244:513–520.
779. Xu J, Mendez E, Caron PR, et al. Bovine viral diarrhea virus NS3 serine proticnase: Polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. *J Virol* 1997;71:5312–5322.
780. Xue W, Minocha HC. Identification of the cell surface receptor for bovine viral diarrhoea virus by using anti-idiotypic antibodies. *J Gen Virol* 1993;74:73–79.
781. Xue W, Zhang S, Minocha HC. Characterization of a putative receptor protein for bovine viral diarrhea virus. *Vet Microbiol* 1997;57: 105–118.
782. Yamada N, Tanihara K, Mizokami M, et al. Full-length sequence of the genome of hepatitis C virus type 3a: Comparative study with different genotypes. *J Gen Virol* 1994;75:3279–3284.
783. Yamada N, Tanihara K, Takada A, et al. Genetic organization and diversity of the 3′ noncoding region of the hepatitis C virus genome. *Virology* 1996;223:255–261.

784. Yamashita T, Kaneko S, Shirota Y, et al. RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. *J Biol Chem* 1998;273: 15479–15486.
785. Yamshchikov VF, Compans RW. Regulation of the late events in flavivirus protein processing and maturation. *Virology* 1993;192:38–51.
786. Yamshchikov VF, Compans RW. Processing of the intracellular form of the West Nile virus capsid protein by the viral NS2B-NS3 protease: An *in vitro* study. *J Virol* 1994;68:5765–5771.
787. Yamshchikov VF, Compans RW. Formation of the flavivirus envelope: Role of the viral NS2B-NS3 protease. *J Virol* 1995;69:1995–2003.
788. Yamshchikov VF, Trent DW, Compans RW. Upregulation of signalase processing and induction of prM-E secretion by the flavivirus NS2B-NS3 protease: Roles of protease components. *J Virol* 1997;71: 4364–4371.
789. Yan Y, Li Y, Munshi S, et al. Complex of NS3 protease and NS4A peptide of BK strain hepatitis C virus: A 2.2 Å resolution structure in a hexagonal crystal form. *Protein Sci* 1998;7:837–847.
790. Yanagi M, Purcell RH, Emerson SU, Bukh J. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci U S A* 1997;94:8738–8743.
791. Yanagi M, Purcell RH, Emerson SU, Bukh J. Hepatitis C virus: An infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* 1999;262: 250–263.
792. Yanagi M, St. Claire M, Emerson SU, et al. *In vivo* analysis of the 3′ untranslated region of the hepatitis C virus after *in vitro* mutagenesis of an infectious cDNA clone. *Proc Natl Acad Sci U S A* 1999;96: 2291–2295.
793. Yanagi M, St. Claire M, Shapiro M, et al. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious *in vivo*. *Virology* 1998;244:161–172.
794. Yao N, Hesson T, Cable M, et al. Structure of the hepatitis C virus RNA helicase domain. *Nat Struct Biol* 1997;4:463–467.
795. Yao N, Reichert P, Taremi SS, et al. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. *Structure Fold Des* 1999;7: 1353–1363.
796. Yasui K, Wakita T, Tsukiyama-Kohara K, et al. The native form and maturation process of hepatitis C virus core protein. *J Virol* 1998;72: 6048–6055.
797. Yen J-H, Chang SC, Hu C-R, et al. Cellular proteins specifically bind to the 5′-noncoding region of hepatitis C virus RNA. *Virology* 1995; 208:723–732.
798. Yi M, Kaneko S, Yu DY, Murakami S. Hepatitis C virus envelope proteins bind lactoferrin. *J Virol* 1997;71:5997–6002.
799. Yoo BJ, Selby M, Choe J, et al. Transfection of a differentiated human hepatoma cell line (Huh7) with *in vitro*-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *J Virol* 1995;69:32–38.
800. Yoo BJ, Spaete RR, Geballe AP, et al. 5′ end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5′ untranslated region. *Virology* 1992;191:889–899.
801. Yoshiba M, Inoue K, Sekiyama K. Hepatitis GB virus C [letter]. *N Engl J Med* 1996;335:1392–1393.
802. Yoshiba M, Okamoto H, Mishiro S. Detection of the GBV-C hepatitis virus genome in serum from patients with fulminant hepatitis of unknown aetiology [see comments]. *Lancet* 1995;346:1131–1132.
803. You S, Padmanabhan R. A novel *in vitro* replication system for dengue virus. Initiation of RNA synthesis at the 3′-end of exogenous viral RNA templates requires 5′- and 3′-terminal complementary sequence motifs of the viral RNA. *J Biol Chem* 1999;274:33714–33722.
804. Yu H, Grassmann CW, Behrens SE. Sequence and structural elements at the 3′ terminus of bovine viral diarrhea virus genomic RNA: Functional role during RNA replication. *J Virol* 1999;73:3638–3648.
805. Yuan Z-H, Kumar U, Thomas HC, et al. Expression, purification, and partial characterization of HCV RNA polymerase. *Biochem Biophys Res Commun* 1997;232:231–235.
806. Yuasa T, Ishikawa G, Manabe S, et al. The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. *J Gen Virol* 1991;72:2021–2024.
807. Zanotto PM, Gould EA, Gao GF, et al. Population dynamics of flaviviruses revealed by molecular phylogenies [see comments]. *Proc Natl Acad Sci U S A* 1996;93:548–553.
808. Zeng L, Falgout B, Markoff L. Identification of specific nucleotide sequences within the conserved 3′-SL in the dengue type 2 virus genome required for replication. *J Virol* 1998;72:7510–7522.
809. Zhang G, Aldridge S, Clarke MC, McCauley JW. Cell death induced by cytopathic bovine viral diarrhoea virus is mediated by apoptosis. *J Gen Virol* 1996;77:1677–1681.
810. Zhang L, Mohan PM, Padmanabhan R. Processing and localization of dengue virus type 2 polyprotein precursor NS3-NS4A-NS4B-NS5. *J Virol* 1992;66:7549–7554.
811. Zhao B, Mackow E, Buckler-White A, et al. Cloning full length dengue 4 viral DNA sequences: Analysis of genes coding for structural proteins. *Virology* 1986;155:77–88.
812. Zhong W, Gutshall LL, Del Vecchio AM. Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus. *J Virol* 1998;72:9365–9369.
813. Zhong W, Ingravallo P, Wright-Minogue J, et al. Nucleoside triphosphatase and RNA helicase activities associated with GB virus B nonstructural protein 3. *Virology* 1999;261:216–226.
814. Zhong W, Uss AS, Fermin E, et al. De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. *J Virol* 2000;74:2017–2022.
815. Zhu N, Khoshnan A, Schneider R, et al. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J Virol* 1998;72:3691–3697.
816. Zitzmann N, Mehta AS, Carrouee S, et al. Imino sugars inhibit the formation and secretion of bovine viral diarrhea virus, a pestivirus model of hepatitis C virus: Implications for the development of broad spectrum anti-hepatitis virus agents. *Proc Natl Acad Sci U S A* 1999;96: 11878–11882.